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(54) Title: COMPOSITIONS AND METHODS FOR BIODETECTION BY NUCLEIC ACID-TEMPLATED CHEMISTRY

(57) Abstract: The invention provides compositions and methods for the detection of biological targets, (*e.g.* nucleic acids and proteins) by nucleic acid-templated chemistry, for example, by generating fluorescent polymethine dyes.



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COMPOSITIONS AND METHODS FOR BIODETECTION BY NUCLEIC ACID-TEMPLATED CHEMISTRY

RELATED APPLICATIONS

[0001] This application claims the benefit of and priority to U.S. Patent Applications Serial Nos. 60/847,859, filed September 28, 2006, 60/905,364, filed March 7, 2007; and 60/918,023, filed March 14, 2007, and PCT International Application No. PCT/US07/_____, titled “Receptor Family Profiling,” by Landsman *et al.*, filed September 18, 2007 in the U.S.

5 Receiving Office, the entire disclosure of each of which is incorporated by reference herein for all purposes.

FIELD OF THE INVENTION

[0002] The present invention relates generally to probes and their use in biodetection and diagnostics. More particularly, the invention relates to compositions and methods for
10 biodetection using nucleic acid-templated chemistry (e.g., synthesis of compounds having desired fluorescent, chemiluminescent or chromophoric properties in a multiplex detection of nucleic acids or proteins).

BACKGROUND

[0003] Fluorescent and colored compounds have been used in the fields of biological
15 research and medicine to detect the presence, absence, state, quantity, and composition of biomolecules. Assays using fluorescent and colored compounds may be performed *in vitro*, *in situ*, or *in vivo*. Examples of commonly used *in vitro* assays for detection of DNA and RNA are real-time and end-point polymerase chain reaction (PCR), DNA sequencing, and DNA microarray technologies.

20 [0004] Recently, there has been an increased amount of literature published on detection methods for multiple analytes, most of which involves genetic analysis and some relates to protein detection. See, e.g., U.S. Patent No. 6,890,741.

[0005] In a typical nucleic acid detection method used for diagnostic and molecular biology research, multiple gene probes complementary with a gene of interest are labeled with small
25 molecules that can be detected by spectroscopic, electrochemical, biochemical or

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immunochemical means. PCR is generally incorporated for the amplification of targeted gene sequences. To achieve detection of multiple analytes, fluorescence-based technologies have been used often due to fluorescence dyes readily available. For example, primers have been labeled with different fluorescence dyes and the changes in fluorescence were monitored upon hybridization to their complements (e.g., WO 2002/057479). In other cases, the multiplex detection was achieved by using intercalating dyes as labels in DNA restriction fragment analysis and capillary electrophoresis with frequency-domain fluorescence lifetime detection method (McIntosh, et al., *Electrophoresis*, **2002**, *23*, 1473-1479). Since these methods use pre-labeled fluorescence dyes, the detection sensitivity relies largely on the separation of target bound and unbound fluorescence labeled probes. Though solid phase immobilization of the target gene (fluorescence *in situ* hybridization, for example) can improve the separation efficiency by simply washing away the unbound fluorescence labeled probes, this introduces an extra process. However, the potential background still can be high, and the procedure can be laborious. To address this problem, a non-fluorescence label moiety can be attached to the probes so that the fluorescence signal only occurs after the hybridization event. Recently, the development of DNA-programmed chemistry has provided a novel approach for generation of fluorescence dye *in situ*. See, e.g., Li, X.; Liu, D. R. *Angew. Chem. Int. Ed.* **2004**, *43*, 4848–4870; U.S. Patent No. 7,070,928.

[0006] Polymethine dye has been widely used as laser dyes, photographic sensitizers and fluorescence probes due to its superior fluorescence and photochemical properties. However, polymethine dyes are generally synthesized by acid/base catalyzed condensation under anhydrous conditions which is not comparable to the nucleic acid-templated chemistry (Jedrzejewska, et al. *Dyes and Pigments* **2003**, *58*, 47–58). Recently, the literature has reported an improved aldol condensation in water using Lewis-acid (Kobayashi, et al., *J. Am. Chem. Soc.* **1998**, *120*, 8287–8288) and enamine-based organocatalyst (Mase, et al. *J. Am. Chem. Soc.* **2006**, *128*, 734–735). The quaternary salt of polymethine precursor (active hydrogen component) used for condensing with aldehyde, however, is different substantially from the precursor (alpha carbon of aldehyde) in a conventional aldol condensation.

[0007] Thus, there exists a need for new fluorescent and colorimetric technologies that address many of the shortcomings inherent in the above-mentioned biodetection methods. For example, there is a need for methods of polymethine dye synthesis from non-detectable

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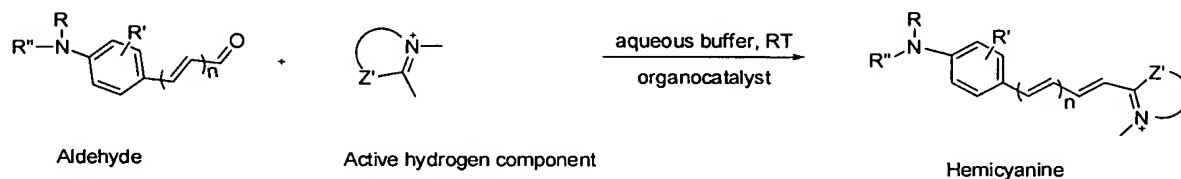
precursors by nucleic acid-templated chemistry and adaptation of such chemistry to biodetection.

SUMMARY OF THE INVENTION

[0008] The present invention is based, in part, upon the discovery that nucleic acid-templated chemistry can be applied in detection of multiple biological targets simultaneously. The present invention is based, in part, upon the discovery that polymethine dyes can be synthesized by nucleic acid-templated chemistry. Assays of this invention may be performed *in vitro*, *in situ*, or *in vivo*.

[0009] In one aspect, the present invention relates to a method for making a polymethine dye comprising conducting an aldol condensation between an aldehyde and an active hydrogen component in an aqueous condition in the presence of an organocatalyst.

[0010] In some embodiments, the condensation reaction is:



wherein

Z' = O, S, Se, P, NH₂, NR, C(CH₃)₂ where R is alkyl group

n = 0, 1, 2 ...

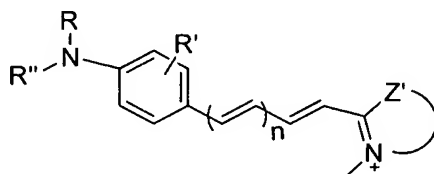
R = H, alkyl

R'' = H, alkyl, alkyl carboxylic acid

R' = Ph or N-heterocycle, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂, NH₂, N(R)₂, OR where R is alkyl group

and wherein the organocatalyst is a secondary amine, a primary amine, a bifunctional amine-acid catalyst or a diamine. The secondary amine may be a pyrrolidine, a piperidine, a normicotine, or an analog thereof, for example. The primary amine may be a valine or a peptide having fewer than 3 amino acid units, for example. The bifunctional amine-acid catalyst may be pyrrolidine/AcOH, for example. The diamine catalyst may be N1,N1-dimethylethane-1,2-diamine, propane-1,2-diamine, 1-(2-aminoethyl)-piperidine, or an analog thereof, for example.

[0011] In another aspect, the invention generally relates to a hemicyanine dye having the chemical structure of (I), (II) or (III), for example, prepared by the methods disclosed herein.



(I)

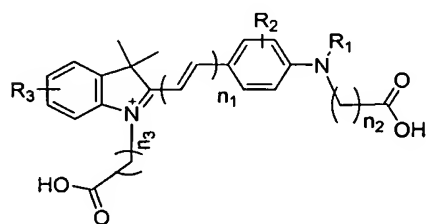
Z' = O, S, Se, P, NH₂, NR, C(CH₃)₂ where R is alkyl group

$$n = 0, 1, 2, \dots$$

R = H, alkyl

R' = H, alkyl
R'' = H, alkyl, alkyl carboxylic acid

R' = Ph or *N*-heterocycle, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂, NH₂, N(R)₂, OR where R is alkyl group



$n1 = 1, 2, 3, 4, 5$

n2, n3 = 0 to 16

R1 = alkyl

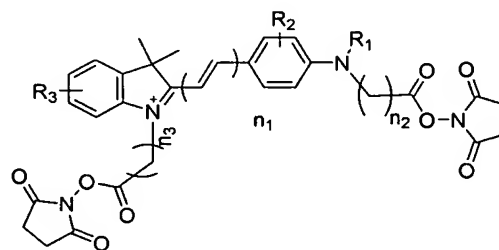
R2 = Ph, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂,

NH₂, N(R₁)₂, OR₁ while R₁ is alkyl group

$R_3 = \text{Ph, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2,$

NH_2 , $\text{N}(\text{R}_1)_2$, OR_1 while R_1 is alkyl group

(II)


$$n_1 = 1, 2, 3, 4, 5$$

n2, n3 = 0 to 16

R1 = alkyl

R² = Ph, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂,

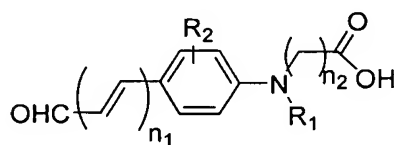
NH₂, N(R₁)₂, OR₁ while R₁ is alkyl group

$R_3 = \text{Ph, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2,$

NH₂, N(R₁)₂, OR₁ while R₁ is alkyl group

(III)

[0012] In yet another aspect, the invention generally relates to an aldehyde having the chemical structure of IV or V:


$$n1 = 1, 2, 3, 4, 5$$

n2 = 0 to 16

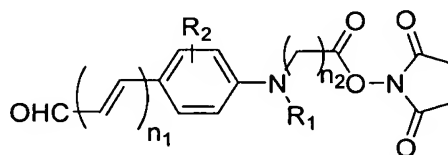
R1 = alkyl

R₂ = Ph, H, alkyl, SO₃H, OH, CN, Cl, Br.

$R_2 = H, H, \text{alkyl}, SO_3H, OH, CN, Cl, Br, NO_2, NH_2, N(R_1)_2, OR_1$ while R_1 is alkyl

NO₂,
group

(IV)


$$n1 = 1, 2, 3, 4, 5$$

$n_2 = 0$ to 16

R1 = alkyl

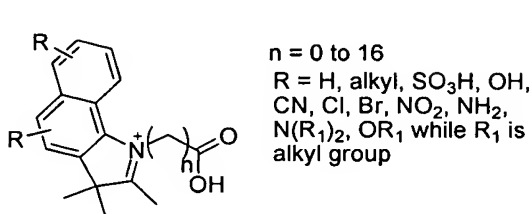
R₂ = Ph, H, alkyl, SO₃H, OH, CN, Cl, Br,

NO₂, NH₂, N(R₁)₂, OR₁ while R₁ is alkyl group

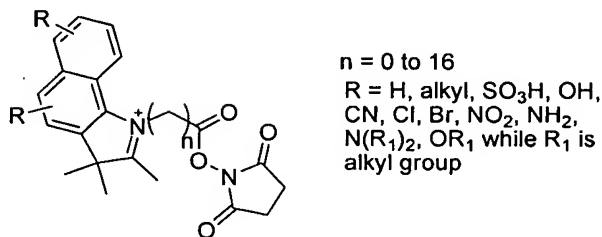
(V)

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[0013] In yet another aspect, the invention generally relates to a quaternary salt having the chemical structure of VI or VII:

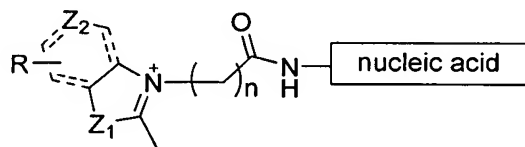


(VI)



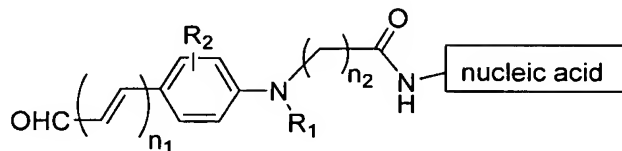
(VII)

5 [0014] In yet another aspect, the invention generally relates to an quaternary salt-nucleic acid conjugate having the chemical structure of:



$n = 0$ to 16
 $\text{Z}_1 = \text{O, S, Se, P, NH}_2, \text{NR}_1, \text{C(CH}_3)_2$ where R_1 is alkyl group
 $R = \text{any substituted benzyl or higher fused benzyl rings, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1$ while R_1 is alkyl group
 $\text{Z}_2 = \text{benzene or any N-heterocycles}$

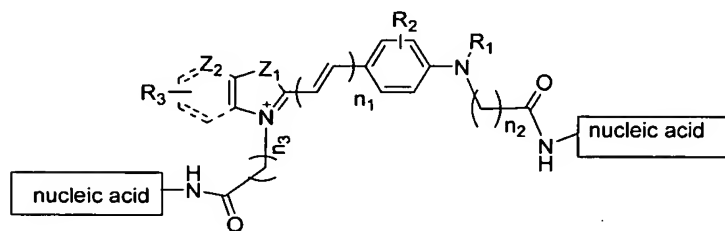
[0015] In yet another aspect, the invention generally relates to an aldehyde-nucleic acid conjugate having the chemical structure of:



$n_1 = 1, 2, 3, 4, 5$
 $n_2 = 0$ to 16
 $\text{R}_1 = \text{H, alkyl}$
 $\text{R}_2 = \text{Ph or N-heterocycle, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1$ while R_1 is alkyl group

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[0016] In yet another aspect, the invention generally relates to a hemicyanine dye-nucleic acid conjugate having the chemical structure of:



$n_1 = 1, 2, 3, 4, 5$
 n_2 and $n_3 = 0$ to 16
 $Z_1 = O, S, Se, P, NH_2, NR_1, C(CH_3)_2$
 where R_1 is alkyl group
 $R_3 =$ any substituted benzyl or higher fused benzyl rings, H, alkyl, SO_3H , OH, CN, Cl, Br, NO_2 , NH_2 , $N(R_1)_2$, OR_1 while R_1 is alkyl group
 $Z_2 =$ benzene or any N-heterocycles
 $R_1 =$ H, alkyl
 $R_2 =$ Ph or N-heterocycle, H, alkyl, SO_3H , OH, CN, Cl, Br, NO_2 , NH_2 , $N(R_1)_2$, OR_1 while R_1 is alkyl group

[0017] In yet another aspect, the invention generally relates to making a hemicyanine-nucleic acid conjugate comprising conducting a nucleic acid-templated reaction between an aldehyde and quaternary salt disclosed herein to make a hemicyanine disclosed herein.

[0018] In some embodiments, the nucleic acid-templated reaction is in an end of helix architect. In some other embodiments, the nucleic acid-templated reaction is in a middle of helix architect.

[0019] In yet another aspect, the invention generally relates to a method for selecting a dye having a desired fluorescent property. The method includes (a) preparing a library of oligonucleotide-encoded dyes through nucleic acid-templated synthesis; (b) hybridizing the oligonucleotide-encoded dyes with spatially arrayed complementary oligonucleotide probes immobilized on a solid support; (c) measuring the absorption and fluorescence properties of the oligonucleotide-encoded dye directly on the solid support; (d) identifying the oligonucleotides that encode the dyes having the desired fluorescence properties based on the position of the immobilized complementary oligonucleotide probes, and (e) identifying and characterizing the chemical structure of the dyes having the desired fluorescence property.

[0020] In yet another aspect, the invention generally relates to a method for detecting multiple target nucleotide sequences. The method includes: (a) providing a number of probe pairs, the number equal to the number of target nucleotide sequences, wherein each probe pair comprises (1) a first probe comprising (i) a first oligonucleotide sequence and (ii) a first reactive group linked to the first oligonucleotide sequence, and (2) a corresponding second probe comprising (i) a second oligonucleotide sequence and (ii) a second reactive group linked to the second oligonucleotide sequence, wherein the first oligonucleotide sequence and the

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second oligonucleotide sequence are complementary to two separate regions of a corresponding target nucleotide sequence; (b) combining the probe pairs with a sample to be tested for the presence of the target nucleotide sequences under conditions where the first probes and the second probes hybridize to their respective complementary regions of the target nucleotide sequences if present in the sample thereby bringing into reactive proximity the first reactive groups and the corresponding second reactive groups; and (c) detecting one or more reactions between the first reactive groups and the corresponding second reactive groups thereby determining the presence of the target nucleotide sequences.

[0021] The number of target nucleotide sequences may be between about 2 to about 20, for example, 2 to 6. The target nucleotide sequences may be in solution phase. The target nucleotide sequences may be attached to a solid support. In some embodiments, the one or more reactions between the first reactive groups and the corresponding second reactive groups generate fluorescent compounds that may be detected. In some embodiment, the one or more reactions between the first reactive groups and the corresponding second reactive groups generate chemiluminescent compounds that may be detected.

[0022] The one or more reactions between the first reactive groups and the corresponding second reactive groups may comprise an aldol condensation reaction, for example. The one or more reactions between the first reactive groups and the corresponding second reactive groups may comprise a Wittig reaction.

[0023] The invention encompasses a kit that provides one, two or more of the probes described herein. More particularly, the invention encompasses a kit that provides one, two or more of the probes that utilize nucleic acid-templated chemistry for the generation of detectable signals as a way for detecting the presence of biological targets.

[0024] The foregoing aspects and embodiments of the invention may be more fully understood by reference to the following figures, detailed description and claims.

DEFINITIONS

[0025] The term, "DNA programmed chemistry" or "DPC", as used herein, refers to nucleic acid-templated chemistry, for example, sequence specific control of chemical reactants to yield specific products accomplished by (1) providing one or more templates, which have associated reactive group(s); (2) contacting one or more transfer groups (reagents) having an anti-codon

(e.g., complementary sequence with one or more templates) and reactive group(s) under conditions to allow for hybridization to the templates and (3) reaction of the reactive groups to yield products. For example, in a one-step nucleic acid-templated reaction, hybridization of a “template” and a “complementary” oligonucleotide bring together reactive groups followed by a chemical reaction that results in the desired product. Structures of the reactants and products need not be related to those of the nucleic acids comprising the template and transfer group oligonucleotides. See, e.g., U.S. Patent No. 7,070,928 and U.S. Application Publication No. 2004/0180412 A1, by Liu *et al.*; Gartner, *et al.*, 2004, Science, vol. 305, pp. 1601-1605; Doyon, *et al.*, 2003, JACS, vol. 125, pp. 12372-12373, all of which are expressly incorporated herein by reference in their entireties. See, also, “Turn Over Probes and Use Thereof” by Coull *et al.*, PCT International Patent Application PCT/US06/16999, filed on May 3, 2006; U.S. patent Application Serial No. 11/441,804, “Biodetection by Nucleic Acid-Templated Chemistry” by Coull *et al.*, filed on May 26, 2006.

[0026] The terms, “nucleic acid”, “oligonucleotide” (sometimes simply referred to as “oligo”) or “polynucleotide” or as used herein refer to a polymer of nucleotides. The polymer may include, without limitation, natural nucleosides (i.e., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine), nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine), chemically modified bases, biologically modified bases (e.g., methylated bases), intercalated bases, modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose), or modified phosphate groups (e.g., phosphorothioates and 5'-N-phosphoramidite linkages).

Nucleic acids and oligonucleotides may also include other polymers of bases having a modified backbone, such as a locked nucleic acid (LNA), a peptide nucleic acid (PNA), a threose nucleic acid (TNA).

[0027] Throughout the description, where compositions are described as having, including, or comprising specific components, or where processes are described as having, including, or comprising specific process steps, it is contemplated that compositions of the present invention also consist essentially of, or consist of, the recited components, and that the processes of the

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present invention also consist essentially of, or consist of, the recited processing steps. Further, it should be understood that the order of steps or order for performing certain actions are immaterial so long as the invention remains operable. Moreover, two or more steps or actions may be conducted simultaneously.

5

BRIEF DESCRIPTION OF THE FIGURES

The invention may be further understood from the following figures in which:

[0028] FIG. 1 illustrates the general chemical structures of polymethine, cyanine and hemicyanine dyes.

10

[0029] FIG. 2 illustrates the general chemical structures of hemicyanine dyes useful for multiplex and their aldehyde and quaternary salt precursors.

[0030] FIG. 3 illustrates the chemical structures of a four-plex hemicyanine-DNA dye system and their spectroscopic properties.

[0031] FIG. 4 is a schematic representation of solution phase-based DPC fluorescence assay for multiple analytes.

15

[0032] FIG. 5 is a schematic representation of solid phase-based DPC fluorescence assay for multiple analytes.

[0033] FIG. 6 is a schematic representation of immunohistochemistry test for multiple family receptor dimers in non-zip-coded architecture.

20

[0034] FIG. 7 is a schematic representation of immunohistochemistry test for multiple family receptor dimers in zip-coded architecture.

[0035] FIG. 8 is a schematic representation of a method for the detection of a biological target under one embodiment of the present invention.

[0036] FIG. 9 is a schematic representation of a method for the detection of a biological target under one embodiment of the present invention.

25

[0037] FIG. 10 shows examples of hybridization as affected by concentration, temperature, and the presence or absence of a single base pair mismatch.

- 10 -

[0038] FIG. 11 shows exemplary oligonucleotides used in certain melting curve experiments.

[0039] FIG. 12 is a schematic representation of a method for the detection of a biological target under one embodiment of the present invention.

5 [0040] FIG. 13 is a schematic representation of a method for the detection of platelet derived growth factor (PDGF) under one embodiment of the present invention.

[0041] FIG. 14 shows exemplary embodiment of a splinted, zip-coded detection system with aptamers as target binding moieties.

10 [0042] FIG. 15 shows exemplary embodiment of a splinted, zip-coded detection system with antibodies as target binding moieties.

[0043] FIG. 16 shows exemplary embodiment of a splinted, zip-coded detection system with antibodies as target binding moieties.

[0044] FIG. 17 shows absorption and fluorescence emission spectra of DPC reaction mixtures (end of helix).

15 [0045] FIG. 18 shows absorption and fluorescence emission spectra of DPC reaction mixtures (end of helix).

[0046] FIG. 19 shows LC-MS data of a crude DPC reaction mixture.

[0047] FIG. 20 shows LC-MS data of a DPC reaction product.

20 [0048] FIG. 21 shows absorption and fluorescence emission spectra of DPC reaction mixtures.

[0049] FIG. 22 shows LC-MS data of a DPC reaction product.

[0050] FIG. 23 shows certain fluorescence intensity data of a DPC reaction.

[0051] FIG. 24 shows certain fluorescence intensity data of a DPC reaction.

25 [0052] FIG. 25 shows fluorescence excitation and emission spectra of four hemicyanine dyes.

[0053] FIG. 26 shows exemplary DNA sequences useful for four-plex hemicyanine dye generation.

[0054] FIG. 27 shows normalized fluorescence emission spectra of four DNA conjugated hemicyanine dyes from middle of helix DPC reactions.

5 [0055] FIG. 28 shows fluorescence kinetic analysis of two DPC reactions in end of helix architecture.

[0056] FIG. 29 shows an example of fluorescence signal generation and biological target detection via triphenylphosphine (TPP) and azidocoumarin (AzC) reporter chemistry.

10 [0057] FIG. 30 shows an example of fluorescence signal generation and biological target detection via TPP and AzC reporter chemistry.

[0058] FIG. 31 shows certain examples of melt curves illustrating the effect of oligonucleotide concentration on T_m .

[0059] FIG. 32 shows certain examples with DNA hybridization melting curves of biotinylated oligonucleotides with and without avidin.

15 [0060] FIG. 33 shows certain examples of T_m changes of complementary biotinylated oligos upon binding to avidin.

[0061] FIG. 34 shows certain examples of the effect of salt and magnesium concentrations upon T_m of oligonucleotides +/- biotin.

20 [0062] FIG. 35 shows certain examples of the melting temperature behavior of biotinylated oligonucleotides at different ratios of oligonucleotides to avidin.

[0063] FIG. 36 shows certain examples of melting curves of 5' and 3' (-) biotin-strand oligos duplexed with biotin-5' (+) strand oligo in the absence and presence of avidin.

[0064] FIG. 37 shows certain examples of melting curves of AT-rich biotinylated oligo dimers with and without avidin.

25 [0065] FIG. 38 is a schematic representation of a method for the detection of a biological target under one embodiment of the present invention.

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[0066] FIG. 39 shows examples of experimental results on detection of a biological target under one embodiment of the present invention.

[0067] FIG. 40A and FIG. 40B show examples of experimental results (the effect of formamide in the reaction mixture) on detection of a biological target under one embodiment of the present invention.

[0068] FIG. 41A and FIG. 41B show examples of experimental results (the effect of formamide in the reaction mixture) on detection of a biological target under one embodiment of the present invention.

[0069] FIG. 42 shows examples of experimental results (the effect of formamide in the reaction mixture) on detection of a biological target under one embodiment of the present invention.

[0070] FIG. 43 shows examples of experimental results (time course of reaction mixtures) on detection of a biological target under one embodiment of the present invention.

[0071] FIG. 44 shows examples of experimental results (time course of reaction mixtures) on detection of a biological target under one embodiment of the present invention.

[0072] FIG. 45 shows examples of experimental results (probe ratios) on detection of a biological target under one embodiment of the present invention.

[0073] FIG. 46 shows an example of detection of PDGF by a zip-coded detection system.

[0074] FIG. 47 shows experiments on ratios of aptamers and reporters.

[0075] FIG. 48 illustrates an embodiment of a "one-piece" detection system for the detection of PDGF.

DETAILED DESCRIPTION OF THE INVENTION

[0076] In its simplest sense, the invention is to detect the presence of target analytes via nucleic acid-templated chemistry, for example, through measurement of fluorescence of polymethine dyes generated by nucleic acid-templated reactions templated by target nucleic acids or proteins. The present invention provides methods for analysis of multiple analytes in a convenient, accurate and sensitive way. For example, in the detection of nucleic acids, the method uses nucleic acid probes conjugated with non-fluorescence precursor (e.g., aldehydes

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and methyl quaternary salts) and polymethine multiplex dyes are generated through the chemical reaction of the probes upon hybridization with target nucleic acids. In addition, the invention provides novel chemical compositions of polymethine dyes and methods of synthesizing polymethine dyes in conventional reactions under aqueous conditions as well as via nucleic acid-templated chemistry.

Polymethine Dye Chemistry

[0077] Polymethine dye is characterized by a chain of methine ($-\text{CH}=\text{}$) groups with an electron donor (D) and an electron acceptor (A) at opposite ends of their polyene chain (FIG. 1, Zollinger, Color Chemistry: Syntheses, Properties, and Applications of Organic Dyes and Pigments, 3rd Edn., Verlag Helvetica Chimica Acta, Postfach, Switzerland, 2003). Typical A and D terminals for polymethine dyes (as shown in FIG. 1) include thiazoles, pyrroles, pyrrolines, indoles, 1, 3, 3-trimethylindolines, tetrazoles, pyrimidine, pyridines, quinolines, and higher fused N-heterocycles or any substituted benzyl rings. If the terminals are both *N*-atom containing heterocycles, the compound is named cyanine. If only one *N*-atom is part of the ring system, the compound is named hemicyanine. By changing the number of the vinyl group in the polyene chain, the fluorescence emission wavelength of the polymethine dye can be tuned from near-UV to near-IR. The terminal group may also provide mean for finer tuning.

[0078] Scheme 1 depicts hemicyanine formation through organocatalytic aldol condensation in aqueous buffer. Some of the general organocatalysts such as pyrrolidine analogues have been listed here. By using catalyst, the reaction condition for the hemicyanine formation can switch from anhydrous to aqueous condition. The percentage of the water content used depends only on the solubility of the starting materials.

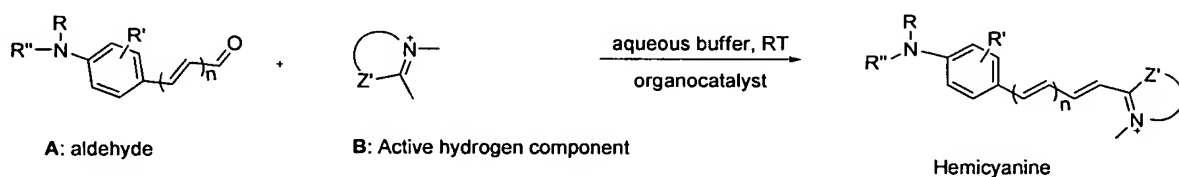
[0079] Scheme 2 is a schematic illustration of hemicyanine dye generation through DPC in the presence of organocatalyst. The general chemical structures of component A (aldehyde_DNA), component B (quaternary salt bearing active hydrogen component) and hemicyanine_DNA conjugate have also been described. The fluorescence emission wavelength of the hemicyanine dye can be tuned by changing the number of the vinyl group in the polyene chain (*n*) or by using different substitute groups (*R'*) and terminal groups in component B.

[0080] FIG. 2 shows the general chemical structures of hemicyanine dyes useful for multiplex and their aldehyde and quaternary salt precursors. An example of four-plex

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hemicyanine_DNA dyes derived from the general structure and their maximum UV absorption and fluorescence's emission wavelength has been described in **FIG. 3**.

Scheme 1: Hemicyanine formation through organocatalytic aldol condensation in aqueous buffer.



Z' = O, S, Se, P, NH₂, NR, C(CH₃)₂ where R is alkyl group

n = 0, 1, 2 ...

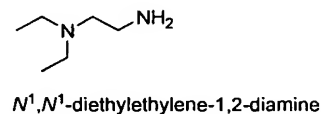
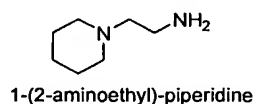
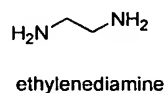
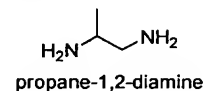
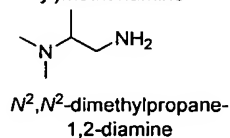
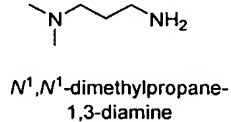
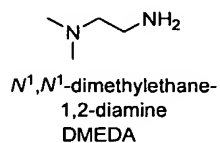
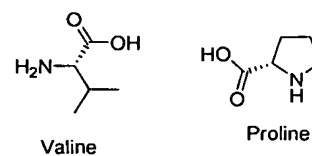
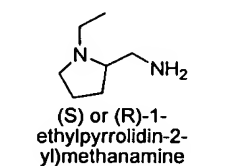
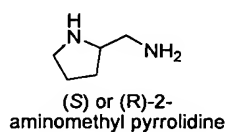
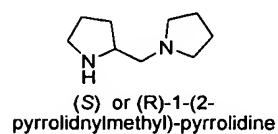
R = H, alkyl

R'' = H, alkyl, alkyl carboxylic acid

R' = Ph or *N*-heterocycle, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂, NH₂, N(R)₂, OR where R is alkyl group

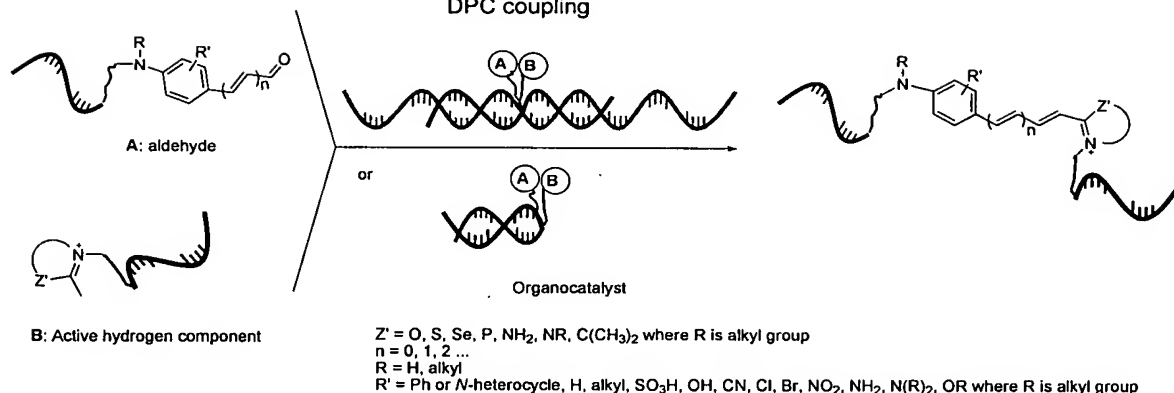
Organocatalysts:

- 1) Pyrrolidine, piperidine analogues
- 2) Amino acid such as valine or small peptide
- 3) Bifunctional amine-acid catalysts such as pyrrolidine/AcOH
- 4) Diamine such as dimethyl-1,2-diamine, propane-1,2-diamine



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Scheme 2: Hemicyanine dye generation through organocatalyst catalyzed DPC.



5 [0081] FIG. 4 is a schematic illustration of solution phase based DPC fluorescence assay for multiplex detection involving multiple analytes. Each pair is labeled with non-fluorescence precursors complementary to a sequence that is indicative of a specific polymorphic site or genotype in the diagnostic determination of infection with a virus which can be PCR-amplified products. After hybridization, DPC fluorescence product will be formed. By changing the

10 hybridization conditions such as salt and temperature, only matched pairs will generate the fluorescence signal. No washing process is needed to remove any non-fluorescence precursors. This method is not only useful for analyzing or detecting polynucleotide sequences, it also can be used, for example, in an antibody based assay utilizing a nucleic acid conjugated antibody.

15 [0082] FIG. 5 is a schematic illustration of a solid phase based DPC fluorescence assay for multiple analytes. Different solid supports can be used to immobilize genes of interest, such as a glass plate, a polymer or a gold plate. After hybridization and fluorescence compound formation by catalyzed DPC, the solid support can be visualized directly by fluorescence microscopy or detected by a fluorescence reader without the need for washing.

20 [0083] FIG. 6 illustrates a multiplexed immunohistochemistry (IHC) test for multiple family receptor dimers. Multiple pairs of probes, each pair being directed at a particular homo- or hetero dimer and with a distinct DPC product (e.g., a distinct fluorescent signal from each pair as shown), can provide simultaneous detection and profiling of multiple receptor dimers. Both zip-coded, as illustrated in FIG. 7, and non-zip-coded probe pairs can be employed in a multiplex test. See, e.g., PCT International Application No. PCT/US07/_____, titled

25 "Receptor Family Profiling," by Landsman *et al.*, filed September 18, 2007 in the U.S. Receiving Office.

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[0084] There are a number of advantages to the detection methods disclosed here. 1) Zero background. There is no background fluorescence signal for aldehyde and quaternary salt precursors when excited at the polymethine product's excitation wavelength. Since the DPC reaction only happens in the presence of the catalyst and the aldehyde and quaternary salt precursors are very stable, no decomposition of precursors will be observed and thus there is no background fluorescence of the decomposed product. 2) Simplicity. The fluorescence generation is performed in one-pot and the detection is achieved *in situ* without isolation of the product. 3) Specificity. The fluorescence generation is based on the sequence specific nucleic acid interaction, so the signal generation is specific to the nucleic acid sequence. 4) A larger number of analytes can be detected as compared to conventional methods. The fluorescence emission wavelength of polymethine dye can be easily tuned from far UV to near IR, so multi-wavelength dyes can be generated in one-pot by utilizing multi-codon DNAs. The numbers of analyte that can be detected are not limited by the DPC and dye chemistry.

[0085] Various and general aspects of nucleic acid-templated chemistry are discussed in detail below. Additional information may be found in U.S. Patent No. 7,070,928, and 7,223,545, European Patent No. 1,423,400 B1 and U.S. Application Publication No. 2004/0180412 A1 (USSN 10/643,752) by Liu *et al.* Gartner, *et al.*, 2004, Science, vol. 305, pp. 1601-1605; Doyon, *et al.*, 2003, JACS, vol. 125, pp. 12372-12373, all of which are expressly incorporated herein by reference in their entireties. See, also, "Turn Over Probes and Use Thereof" by Coull *et al.*, PCT WO07/008276A2, filed on May 3, 2006.

DPC-BASED PROTEIN DETECTION

[0086] Methods and compositions of biodetection using nucleic acid-templated chemistry based probes are described in WO06128138A2 by Coull *et al.*, which is incorporated herein by reference in its entirety.

[0087] FIG. 8 and FIG. 9 illustrate one embodiment of the invention for the detection of a protein target.

[0088] FIG. 8 shows an embodiment of detection of a protein target by DPC-based probes. Two probes contain target binding moieties, complementary oligonucleotides, and chemically reactive species X and Y, respectively. Upon hybridization, X and Y react to create a signal generating (e.g., fluorescent) compound, which may or may not covalently link both probes.

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The reaction product of X and Y may also be released as an unbound, soluble compound into the solution. The protein target may be attached to a solid-phase such as the surface of a bead, glass slide (microarray), etc., or be in solution. The target binding moieties may be aptamers, antibodies, antibody fragments (i.e., Fab), receptor proteins, or small molecules, for example.

5 [0089] More particularly illustrated in FIG. 9 is an example of the dual-probe approach with two probes, each carrying a “prefluorophore” precursor (R1 and R2) and containing a binding moiety for a target and an oligonucleotide sequence that is designed to anneal to each other. In this embodiment, the detection is performed under conditions such that the prefluorophore
10 oligos will not anneal to each other in the absence of a target. These conditions are generally selected such that the ambient temperature is higher than the T_m of the oligonucleotide pairs in the absence of the target (so that the oligo pairs will not anneal in the absence of the intended target analyte). In the presence of the intended target, however, the localized high
15 concentration of the oligos then shifts the T_m of their double stranded complex upwards so that hybridization occurs, which is followed by a signal-generating nucleic acid-templated reaction (a reaction between R1 and R2). The signal-generating nucleic acid-templated reaction is
20 accelerated both due to the localized higher concentration of the prefluorophores, but may also be facilitated by the proximity and orientation of the prefluorophore groups towards one another. This configuration of signal generation has the potential to enable creation of kits for the detection of various biomolecules, cells, surfaces and for the design of *in situ* assays. The
25 signal generation does not require enzymes and the homogeneous format requires no sample manipulation.

[0090] In FIG. 9, two oligonucleotides are shown, each of which is linked through an optional spacer arm to a separate binder, as shown in this case is an antibody but may be other binders such as aptamers or small molecules. Each antibody recognizes a separate epitope on a
25 common target analyte such as a protein. Spacer arms can be added to one or both oligonucleotides between the oligo and the binder. In certain cases, this spacer arm may be required to meet proximity requirements to achieve a desired reactivity. Spacer arms in principle can be any suitable groups, for example, linear or branched aliphatic carbon chains C3 to C5, C10, C15, C20, C25, C30, C35, C40, or C100 groups, a DNA sequence of 1 to 10, 15,
30 20, 30, 50 or 100 bases long, or polyethylene glycol oligomers of the appropriate length.

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[0091] The prefluorophores may reside in an “end of helix” configuration (**FIG. 9** top), one attached to the 5’ end of an oligo and other to the 3’ end. (Other configurations can be applied, including placing the two prefluorophores within the sequence or having one oligo hybridize to a partial hairpin structure (e.g., 100 Angstroms long), for example.) In the first example, one oligonucleotide is attached to a 5’ to a spacer arm and a target binder, and the other 3’ is attached to a spacer arm and separate target binder. Spacer arms, which can consist of non-complementary DNA sequences, or synthetic spacer arms such as oligomers of ethylene glycol, can be added to meet proximity requirements. Such spacer arms can be very flexible, which has the advantage of overcoming any steric hindrance to binding that might occur with a rigid spacer. A suitably long spacer arm design can permit both oligonucleotides to be linked 5’ to their binders (**FIG. 9** bottom), or both linked 3’, as long as the oligonucleotides can anneal in the antiparallel configuration and allow the reactive groups to react with each other. An optimal spacer arm length may be designed for each target. Spacer arms which are excessively long should be avoided as they may reduce specificity in the system or a reduced increased T_m effect.

[0092] The proximity effect afforded by tethering the pair of oligonucleotides may affect the kinetics of annealing of two complementary oligonucleotide sequences compared to the two oligonucleotides free in solution. More importantly, a localized high concentration shifts the melting curve upwards compared to the free complex, i.e. increase the T_m of the complex. In a bulk solution, it is known that T_m has dependence upon total oligonucleotide concentration as illustrated in the equation below. Wetmur, *Crit. Rev. in Biochem. And Mol. Biol.*, **1991**, 26, 227-259.

$$T_m = (1000 * \Delta H) / (A + \Delta S + R \ln(C_t / 4) - 273.15 + 16.6 \log Na^+)$$

where ΔH and ΔS are the enthalpy and entropy for helix formation, R is the molar gas constant, C_t is the total concentration of oligomers, and Na^+ is the molar concentration of sodium ion in the solution.

[0093] **FIG. 10** shows that the slope of T_m vs. concentration within the range of short oligonucleotides in 0.1 M salt has a dependence of about +7° C per 10-fold increase in concentration of oligonucleotides (sequences in **FIG. 11**) based on the above equation. So, for

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example, a 1000-fold increase in local concentration would be expected to raise T_m by about +21° C.

[0094] Reaction products of R1 and R2 may be released from the hybridization complex as a result of the chemical transformation. Thus, the fluorophore or chromophore may be separated
5 from the hybridization complex and analyzed independently, or the fluorophore or chromophore and the annealed oligonucleotides may be removed once detected so that additional rounds of interrogation of the sample can be conducted. The reaction between R1 and R2 may or may not be covalently linked to the two probes once the product(s) is formed.

[0095] FIG. 12 illustrates another embodiment of the invention, which employs a “zip-
10 coded” splint architecture for nucleic acid template-based biodetection. In this embodiment, instead of the target binding moieties being directly linked (optionally via spacer groups) to the complementary oligonucleotides that hybridize and set up nucleic acid templated reactions, the target binding moieties is linked to a “zip code” oligonucleotide sequence. Each of the
15 corresponding reporter oligonucleotides has a complementary, “anti-zip code” sequence (in addition to a “reporter” sequence that sets up nucleic acid-templated reaction). The nucleic acid-templated chemical reactions are set up by the hybridization of the reporter oligos, which are linked to reactive groups that react and generate detectable signals. It is important that each oligonucleotide sequence of the probes is complementary only to its intended hybridization partner and not complementary to other oligonucleotides in the detection system.

[0096] This zip-coded architecture supports creating a single reporter-oligonucleotide
20 conjugate which would assemble with different downstream reporter oligonucleotides through an anti-zip code sequence. Libraries of different reporters linked to a unique anti-zip code may be tested simply by mixing each one with stoichiometric amounts of the binder-zip code oligonucleotide conjugate with its complementary zip code.

[0097] FIG. 13 is an illustration of a zip-coded splinted architecture approach where the
25 target binding moieties are two aptamers. In this example for detection of platelet derived growth factor (PDGF) with illustrative oligo sequences and reporter chemistry (e.g., triphenylphosphine, TPP, and 7-azidocoumarin, AzC), the TPP reporter oligonucleotide self-assembles to the PDGF aptamer oligonucleotide through hybridization of zip code sequence
30 (NNN.....) to the complementary anti zip code sequence (N’N’N’.....) on the TPP reporter

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oligonucleotide. The reporter oligonucleotide terminates with an exemplary 10-base reporter sequence and a 5'-TPP group. A separate pair of oligonucleotides, with different zip codes and anti-zip codes (complementary to each other pairwise), also self-assembles to provide the AzC reporter sequence and a 3'-AzC group. The AzC oligonucleotides are complementary and antiparallel to the TPP oligonucleotides so the TPP and AzC groups terminate end-to-end when the TPP and AzC oligonucleotides anneal to each other.

[0098] FIG. 14 illustrates in more detail the zip-coded splinted architecture approach for detection of PDGF with illustrative oligo sequences and reporter chemistry (TPP and AzC). The TPP pair includes, first, a PDGF-aptamer on the 5'-end, a C18 polyethylene-glycol based spacer, and an 18-mer zip code sequence. The TPP reporter sequence includes a complementary anti-zip code sequence on its 3' terminus, a C18 PEG spacer, and a ten base pair reporter sequence terminating in a 5' TPP group. The AzC pair of oligonucleotides includes a 3'-aptamer linked through a C18 PEG spacer to a separate zip code, and a detection oligonucleotide linked to a 5' anti-zip code, a C18 PEG spacer, and a reporter oligonucleotide (complementary to the TPP oligonucleotide) terminating in a 3' AzC group.

[0099] FIG. 15 illustrates an example of the corresponding architect where antibodies are used instead of aptamers as target binding moieties.

[0100] One advantage of the "zip coded" approach is the ability to create the reporter oligonucleotides separately, and have them assemble together with binders under conditions retaining the activities of both the binders and of the nucleic acid template-activated chemistry.

[0101] The zip-coded system is based upon two pairs of oligonucleotides, with each pair being held together by the base-pairing of a unique zip code and an anti-zip code pair. "Zip codes" are oligonucleotide sequences which bind specifically to their complementary sequences, and preferably are designed such they are not complementary to known genomic sequences (relevant if the sample may contain genomic DNA), have similar T_m values, lack significant secondary structure, and do not anneal to other zip code or anti-zip code sequences in the detection system.

[0102] It is worth pointing out the methods of the invention do not require enzymatic or chemical ligation of the first and/or the second oligonucleotide sequences.

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[0103] Factors that may be considered in optimizing a design of a zip-coded architecture include, for example, (1) spacer groups (e.g., oligonucleotides and/or non-base groups) between the aptamer/antibody and zip codes (spacer 1), e.g., to allow hybridization partners to reach each other, to prevent any steric hindrance; (2) Length of a zip code sequence in order to form a sufficiently stable annealing to the anti-zip code sequence to form the complex; and (3) Spacer groups (spacer 2) between the anti-zip code and the reporter sequence, e.g., to prevent any steric hindrance.

[0104] The binders (target binding moieties) attached to the oligonucleotides may be any chemical moieties that specifically bind to a target molecule and allow the design of the invention to work. Examples include a wide range of functionalities, such as (1) antibodies: e.g., IgG, IgM, IgA, IgE, Fab's, Fab', F(ab)₂, Dab, Fv or ScFv fragments; (2) small molecule binders, such as inhibitors, drugs, cofactors; (3) receptors for protein detection, and vice versa; (4) DNA, RNA, PNA aptamers; (5) DNA sequences for DNA-binding and regulatory proteins; (6) peptides representing protein binding motifs; (7) peptides discovered through phage display, random synthesis, mutagenesis; (8) naturally binding protein pairs and complexes; (9) antigens (for antibody detection); and (10) a single polyclonal antibody separately attached to two oligonucleotides may serve as two separate binders of different specificity.

[0105] The target binding moieties attached to the oligonucleotides may be of heterogeneous types directed against different sites within the same target. For example, the two binders may be two different antibodies, an antibody and a receptor, an antibody and a small molecule binder, a receptor and a peptide, an aptamer and a cofactor, or any other combination.

[0106] The target analytes can be of any type, provided the target supports two (or more) binding sites. Molecules which exist in equilibrium with a monomeric form and a homodimeric or higher polymerization phase may be detected by a pair of probes containing the same binder but different complementary DNA sequences. Suitable targets include proteins, cell surfaces, antibodies, antigens, viruses, bacteria, organic surfaces, membranes, organelles, *in situ* analysis of fixed cells, protein complexes. The invention may be particularly suited for the detection of fusion proteins (e.g., BCR-ABL in the presence of BCR and ABL).

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[0107] In the design of the probes, one consideration is the T_m of the two reporter sequences carrying the reactive groups. Since the T_m of the duplex should be below room temperature in the absence of a target, this sequence normally should be short, for example 6-15 bases and/or A-T rich. A typical reporter length of 10 base pairs might have a T_m of around 30°C at a low salt concentration. Therefore, it is often necessary even with a short sequence to add 10% to 40% volume/volume formamide to further lower the temperature below assay temperature, or to elevate the assay temperature. Very short reporter oligonucleotides may suffer from a lack of specificity and exhibit some binding to zip code sequences (when these are employed) which is undesirable.

[0108] Another factor in the design of the probes is the length of oligonucleotide in between the binding moiety and the reporter sequence, including any zip code sequences. These must be long enough for the reporter oligonucleotides to reach each other and anneal. The sequences may be interspersed with polyethylene glycol (PEG) linkers that are flexible and may afford additional protection against any steric hindrance. For example, total lengths of oligonucleotides may be around 35 bases long. Oligonucleotides containing 0, 1, or 2 C18 PEG spacers, or homopolymer tracts may also be utilized (i.e. C₁₀).

[0109] A third consideration is the length of zip and anti-zip sequences when these are employed (i.e. FIG. 13 and FIG. 16). Aside from the need for each zip code to anneal only to its anti-zip code, and not any other zip code, anti-zip code, or reporter sequence, an important parameter is the T_m of the duplex between the zip codes and anti-zip codes. The T_m should be substantially higher than the highest temperature that will be used in the assay in order that the reporter oligonucleotides remain firmly attached to the binding moiety. In practice, zip codes of about twice the length of the reporter sequences (i.e. total length of 15-30 bases) are desirable and generally meet these criteria.

[0110] Regarding signal generation, nucleic acid-templated chemistry may be used to create or destroy a label that effects an optical signal, e.g., creating or destroying a fluorescent, chemiluminescent, or colorimetric molecule. Additionally, a detection reaction may be designed to create or destroy a product that directly or indirectly creates a detectable label, for example, a product that catalyzes a reaction that creates an optical label; inhibits a reaction that creates an optical label; is a fluorescence quencher; is a fluorescent energy transfer molecule;

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creates a Ramen label; creates an electrochemiluminescent label (i.e. ruthenium bipyridyl); produces an electron spin label molecule.

[0111] The following examples contain important additional information, exemplification and guidance that can be adapted to the practice of this invention in its various embodiments and equivalents thereof. Practice of the invention will be more fully understood from these following examples, which are presented herein for illustrative purpose only, and should not be construed as limiting in anyway.

EXAMPLES

[0112] Examples 1 to 4 are related to DNA probe preparation. Both the aldehyde and heterocyclic precursors bearing an active hydrogen component can be conjugated to DNA through amide bond formation. First, an acid heterocyclic or aromatic precursor is synthesized. The acid is then converted to the active N-hydroxysuccinimide ester (NHS ester) that readily reacts with DNA bearing amine functionality.

[0113] Oligonucleotides were prepared using standard phosphoramidite chemistry and purified by reversed-phase C18 column (Glen Research, Sterling VA, USA). Oligonucleotides bearing 5'-amino groups were prepared using 5'-Amino-Modifier 5 and oligonucleotides bearing 3'-aminogroups were prepared using 3'-Amino-Modifier C7 CPG (Glen Research, Sterling VA, USA). Concentration of the DNA and heterocyclic conjugated DNA was determined by UV absorbance at 260 nm. The contribution of the UV absorbance at 260 nm from the heterocyclic moiety in the heterocyclic conjugated DNA was negligible and was not considered.

Table 2 Oligonucleotide Sequences

Oligo	sequence (5'-3')	ID No.
Zip2	TTGGTGCTCGAGTCCCCCCCCCCCCCCCCCCCC-NH ₂	(SEQ ID NO: 66)
Zip3	NH ₂ -CCCCCCCCCCCCCCCCCCCCGCTGCCATCGATGGT	(SEQ ID NO: 67)
Zip5	NH ₂ -CCCCCCCCCCCCCCCCCCCCGTGCCATCCATAGTCAG	(SEQ ID NO: 68)
Antizip2 reporter1	GGACTCGAGCACCAATAC-X-TATAAATTTCG-NH ₂	(SEQ ID NO: 69)
Antizip3 reporter	NH ₂ -CGAATTTATA-X-CTGACCATCGATGGCAGC	(SEQ ID NO: 70)
Antizip5 reporter mismatch	NH ₂ -CCAATTAATA-X-CTGACTATGGATGGCAGC	(SEQ ID NO: 71)

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Antizip5 reporter NH₂-CGAATTTATA-X-CTGACTATGGATGGCACG (SEQ ID NO: 72)

Antizip2 report2 GGACTCGAGCACCAATACXTATAAAATTCGCCC (SEQ ID NO: 73)

5 X = Spacer Phosphoramidite 18 (Glen Research, Sterling VA, USA)

EDC1 GTGGTAGTTGGAGCTGGTGGCGTAGGCAAG (SEQ ID NO: 74)

EDC2 H₂N-AGCTC CAACTACCAC (SEQ ID NO: 75)

EDC3 H₂N-AGATCCCACTAGCAC (SEQ ID NO: 76)

EDC4 GTGGTAGTT GGAGCT-NH₂ (SEQ ID NO: 77)

10 EDC5 TCTTGCCTACGCCAC -NH₂ (SEQ ID NO: 78)

EDC7 NH₂ -ACCCTTGAACACGTC (SEQ ID NO: 79)

EDC8 TCTCCGTTGCCGCTC-NH₂ (SEQ ID NO: 80)

EDC10 GTGGTAGTTGGAGCTGGAGCGGCAACGGAGA (SEQ ID NO: 81)

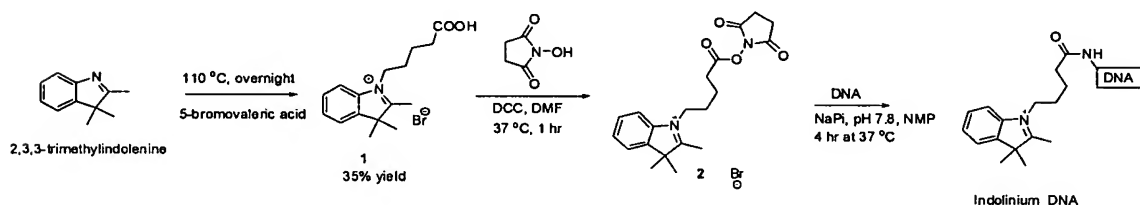
EDC11 GACGTGTTCAAGGGTGGTGGCGTAGGCAAGA (SEQ ID NO: 82)

15 EDC12 GACGTGTTCAAGGGTGGAGCGGCAACGGAGA (SEQ ID NO: 83)

Example 1 DNA conjugated quaternary salt

[0114] Scheme 3 gives one example of synthesizing DNA conjugated quaternary salt bearing active hydrogen component (indolinium_DNA). 2,3,3-trimethylindolenine is commercially available. The acid functionality is introduced to the indoline ring through N-quaternization.

Scheme 3: Example of synthesizing DNA conjugated quaternary salt bearing active hydrogen component (indolinium_DNA).



25 [0115] Synthesis of compound 1: To 5-bromovaleric acid (2.435 g, 13.45 mmole) was added 2,3,3-trimethylindolenine (2.141 g, 13.45 mmole). The reaction mixture was heated with rigorous stirring at 110 °C overnight. The dark red sticky oil obtained was transferred to a Gregar extractor and extracted with EtOAc overnight. A light red solid was obtained. The solid

- 25 -

was redissolved in 30 mL of MeOH. MeOH was removed under reduced pressure and the remaining residue was treated with 10 mL of EtOAc. Browish solid was precipitated out and filtrated. The solid was washed with 2 x 50 mL of acetone and 2 x 100 mL of EtOAc. Total 1.590 g of light brownish solid was obtained (35% yield). ¹H NMR (DMSO) δ_{ppm} : 7.98 (m, 1H), 7.84 (m, 1H), 7.61 (m, 2H), 4.49 (t, 2H), 2.84 (s, 3H), 2.30 (t, 2H), 1.84 (m, 2H), 1.63 (m, 2H), 1.53 (s, 6H). MALDI-MS (positive mode): 260.2419.

[0116] Synthesis of compound 2: Compound 1 (0.1 g, 0.294 mmole), *N*-hydroxy succimide (0.068 g, 0.588 mmole) and *N, N'*-dicyclohexylcarbodiimide (DCC) (0.085 g, 0.411 mmole) were dissolved in 1.5 mL of dimethyl formamide (DMF). The reaction mixture was stirred at 37 °C for 1 hr. The precipitated dicyclohexylurea (DCU) was removed by filtration, and the filtrate was treated with 15 mL of ether. Light orange solid was washed three times with 10 mL of ether and dried under vacuum for several hours. The solid obtained was used directly for the next reaction. MALDI-MS (positive mode): 357.1590.

[0117] Labeling DNA with indolinium compound: To a 1.5 mL of centrifugation vial containing 20 nmole of DNA was added 41.6 μL of 0.1 M sodium phosphate buffer (NaPi), pH 7.8, 41.6 μL of compound 2 in *N*-methyl 2-pyrrolidone (NMP) (96 mM) and 41.6 μL of NMP. The vial was placed in a shaker and shaken for 4 hr at 37 °C. The reaction mixture was desalted by gel filtration using Sephadex G-25 and then purified by reversed-phase C8 column.

Indolinium_EDC2 (DNA: SEQ ID NO: 75): 15% yield. LC-MS (negative mode): Calcd for

$\text{C}_{163}\text{H}_{212}\text{N}_{57}\text{O}_{90}\text{P}_{15}$ (monoisotopic): 1216.7379 $[\text{M}-5\text{H}]^{-4}$; Found: 1216.9552 $[\text{M}-5\text{H}]^{-4}$;

Indolinium_EDC4 (DNA: SEQ ID NO: 77): 22% yield. LC-MS (negative mode): Calcd for $\text{C}_{172}\text{H}_{221}\text{N}_{60}\text{O}_{96}\text{P}_{15}$ (monoisotpic): 1280.5002 $[\text{M}-5\text{H}]^{-4}$; Found: 1280.7356 $[\text{M}-5\text{H}]^{-4}$;

Indolinium_EDC5 (DNA: SEQ ID NO: 78): 15% yield. LC-MS (negative mode): Calcd for $\text{C}_{166}\text{H}_{220}\text{N}_{51}\text{O}_{95}\text{P}_{15}$ (monoisotpic): 1226.7426 $[\text{M}-5\text{H}]^{-4}$; Found: 1226.9657 $[\text{M}-5\text{H}]^{-4}$.

Indolinium_antizip5 (DNA: SEQ ID NO: 72): 10% yield. LC-MS (negative mode): Calcd for $\text{C}_{307}\text{H}_{401}\text{N}_{108}\text{O}_{180}\text{P}_{29}$ (average): 1340.2614 $[\text{M}-8\text{H}]^{7-}$; Found: 1340.2705 $[\text{M}-8\text{H}]^{7-}$. Indolinium_antizip5m (DNA: SEQ ID NO 71): 10% yield. LC-MS (negative mode): Calcd for

$\text{C}_{308}\text{H}_{404}\text{N}_{109}\text{O}_{177}\text{P}_{29}$ (monoisotopic): 1336.4969 $[\text{M}-8\text{H}]^{7-}$; Found: 1336.673 $[\text{M}-8\text{H}]^{7-}$

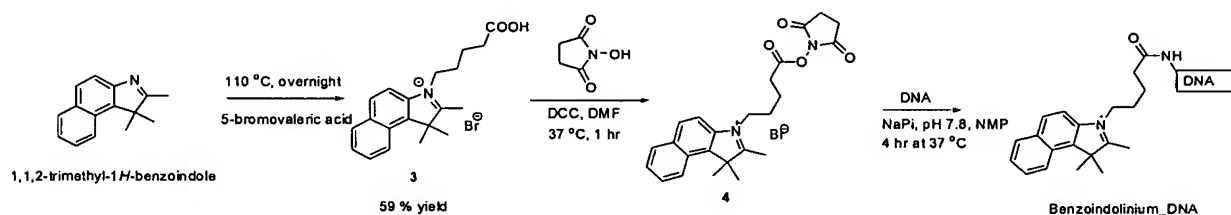
Indolinium_antizip3 (DNA: SEQ ID NO: 70): 5% yield. LC-MS (negative mode): Calcd for

$\text{C}_{307}\text{H}_{404}\text{N}_{107}\text{O}_{178}\text{P}_{29}$ (monoisotopic): 1332.4034 $[\text{M}-8\text{H}]^{7-}$; Found: 1332.6293 $[\text{M}-8\text{H}]^{7-}$.

Example 2 DNA conjugated quaternary salt

[0118] **Scheme 4** provides another example of synthesizing DNA conjugated quaternary salt bearing active hydrogen component (benzoindolinium_DNA) following the similar route as indolinium_DNA.

5 **Scheme 4: Example of synthesizing DNA conjugated quaternary salt bearing active hydrogen component (benzoindolinium_DNA).**



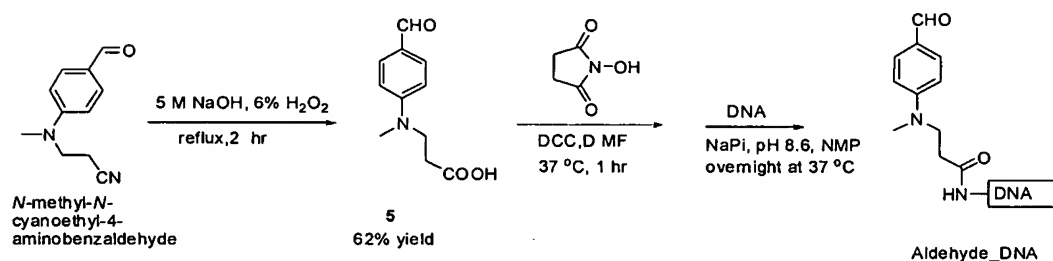
[0119] Synthesis of compound 3: (same procedure of synthesizing compound 1): 1,1,2-trimethyl-1H-benzoinidole (2.73 g, 13 mmole) and 5-bromovaleric acid (2.36 g, 13 mmole) was heated with rigorous stirring at 110 °C overnight. Total 3.016 g of 4 was obtained after working up as off-white solid (59% yield). ¹H NMR (CD₃OD) δ_{ppm}: 8.30 (m), 8.15 (m), 8.05 (d), 7.7 (m), 4.66 (t, 2H), 2.45 (t, 2H), 2.1 (m, 2H), 1.85 (m, 2H), 1.85 (s, 3H), 1.83 (s, 6H). MALDI-MS (positive mode): 310.209.

15 [0120] Synthesis of compound 4: Compound 4 was synthesized following the same procedure of synthesizing compound 2 and was used directly for labeling DNA after ether precipitation.

[0121] Labeling DNA with benzoindolinium compound: Following the same procedure of synthesizing indolinium_DNA, total 11.2 nmole of Benzoindolinium_EDC7 (DNA: SEQ ID NO: 79) was obtained starting from 50 nmole of EDC7: 22% yield. LC-MS (negative mode): Calculated for C₁₆₈H₂₁₆N₅₆O₉₂P₁₅⁺ (monoisotopic): 1650.0003 [M-4H]⁻³; Found: 1650.0359 [M-4H]⁻³.

Example 3 DNA conjugated aldehyde

[0122] **Scheme 5** provides one example of synthesizing DNA conjugated aldehyde. The acid functionality in aldehyde precursor is introduced through hydrolysis of a cyano group by hydrogen peroxide (Brady, J. D.; Robins, S. P. J. Bio. Chem. 2001, 276, 18812-18818.).

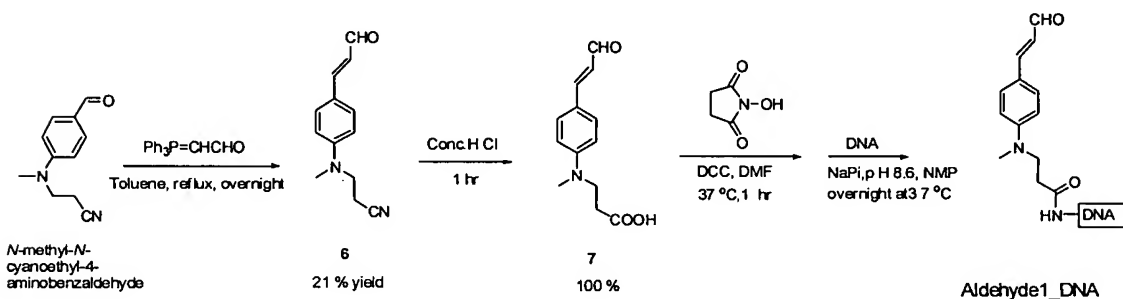
Scheme 5: Example of synthesizing DNA conjugated aldehyde (A0_DNA).

[0123] Synthesis of compound 5: In a 50 mL of round-shaped flask containing *N*-methyl-*N*-cyanoethyl-4-aminobenzaldehyde (1.024 g, 5.44 mmole) was added 27.2 mL of 5 N NaOH solution and 6.8 mL of 30% H₂O₂. The reaction mixture was refluxed for 2 hr. After cooling down, the reaction mixture was neutralized by the addition of concentrated HCl (37% w.t.) and extracted with 2 x 100 mL of EtOAc and 1 x 100 mL of CH₂Cl₂. The organic layers were combined and washed once with 50 mL of brine and concentrated to dryness. The crude product was purified by a 40 g RediSep silica-gel column on a CombiFlash Companion chromatography system (EtOAc/MeOH). Total 0.702 g of light pinkish solid was obtained (62%). Electrospray MS: M+H 208.0735. (Brady, et al., *J. Biol. Chem.* **2001**, 276, 18812–18818).

[0124] Labeling DNA with aldehyde: The NHS ester of 5 was synthesized following the same procedure of compound 2. After removing the DCU by filtration, the filtrate was used directly for DNA conjugation (calculated as 0.2 M product in DMF). To a 1.5 mL of centrifugation vial containing 50 nmole of DNA was added 104 μL of 0.1 M NaPi, pH 8.6, 125 μL of the above filtrate and 83 μL of NMP. The vial was placed in a shaker and shaken overnight at 37 °C. The reaction mixture was desalted by gel filtration using Sephadex G-25 and then purified by reversed-phase C8 column. Aldehyde_EDC2 (DNA: SEQ ID NO: 75) (44% yield). LC-MS: Calcd for C₁₅₈H₂₀₄N₅₇O₉₁P₁₅ (monoisotopic): 1203.9710 [M-4H]⁴⁻; 1605.6306 [M-3H]³⁻ Found: 1203.9664 [M-4H]⁴⁻; 1605.6305 [M-3H]³⁻; Aldehyde_EDC3 (DNA: SEQ ID NO: 76): (49% yield). LC-MS: Calcd for C₁₅₉H₂₀₄N₅₉O₉₁P₁₅ (monoisotopic): 1213.9725 [M-4H]⁴⁻; 1618.9660 [M-3H]³⁻ Found: 1213.9620 [M-4H]⁴⁻; 1618.9590 [M-3H]³⁻. Aldehyde_antizip2 reporter1 (DNA: SEQ ID NO: 69) (30% yield). LC-MS: Calcd for C₃₀₃H₃₉₆N₁₁₀O₁₇₇P₂₉ (monoisotopic): 1328.2458 [M-7H]⁷⁻; Found: 1328.3051 [M-7H]⁷⁻.

Example 4 DNA labeled α,β -unsaturated aldehyde

[0125] Scheme 6 provides an example of synthesizing DNA labeled α,β -unsaturated aldehyde 1. Wittig reagent was used for the two-carbon homologation of aldehydes into the corresponding α,β -enals (Eitel, M.; Pindur, U. *Synthesis* **1989**, 364-367. The acid functionality in aldehyde precursor is introduced through hydrolysis of a cyano group by concentrated HCl (Bratenko, M. K.; Chornous, V. A.; Vovk, M. V. *Chemistry of Heterocyclic Compounds* **2004**, 40, 1279-1282).

Scheme 6: Example of synthesizing DNA conjugated aldehyde (A1_DNA).

[0126] Synthesis of compound 6: In a 100 mL of round-shaped flask containing *N*-methyl-*N*-cyanoethyl-4-aminobenzaldehyde (1.116 g, 5.9 mmole) and ylide (2.71 g, 8.9 mmole) was added 57 mL of dry toluene. The reaction mixture is heated under reflux for overnight, allowed to cool, and filtered through filter paper. After removing the solvent from the filtrate, the residue was first purified by a 40 g RediSep silica-gel column on a CombiFlash Companion chromatography system (Toluene/Ether) and then Preparative HPLC C18 column (Agilent Prep-C18, 30 x 100 mm, 10 μm) to afford 0.27 g of pure product (21%). MALDI-MS (positive mode): 215.226.

[0127] Synthesis of compound 7: In a 50 mL of round-shpaed flask containing compound 6 (0.1 g, 0.47 mmole) was added 30 mL of concentrated HCl. The reaction mixture was heating to boiling and left at room temperature (RT) for 1 hr. HPLC analysis indicated that only one product was formed and no starting material remained in the reaction mixture. After removing most of the HCl, the compound was dissolved in water and lyopholyzed to dryness to afford the product.

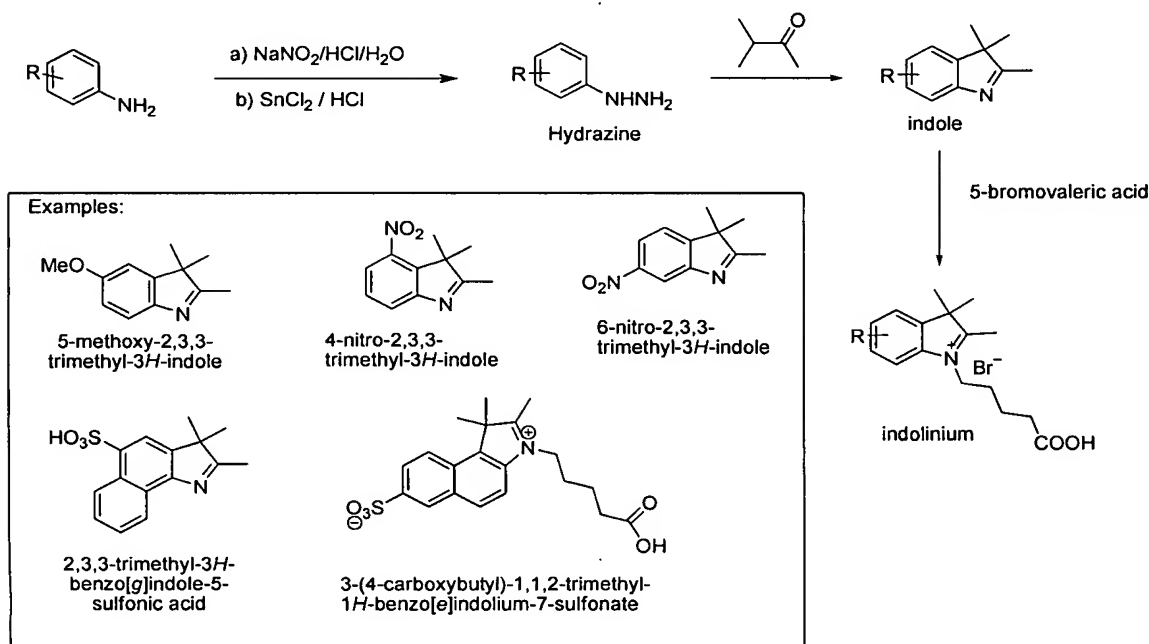
[0128] Labeling DNA with α,β -unsaturated aldehyde: The NHS ester of compound 7 was synthesized following the same procedure as compound 2, however was purified by silica-gel chromatography (EtOAc/Hexanes) instead. After drying under vacuum for several hours, the NHS ester of compound 7 was dissolved in NMP (96 mM) and was used to label DNA

following the same procedure as labeling aldehyde_DNA. Aldehyde1_EDC8 (DNA: SEQ ID NO: 80): yield 46%. Calcd for $C_{163}H_{215}N_{48}O_{99}P_{15}$ (monoisotopic): 1629.9698 [M-3H]⁻³; Found: 1629.9995 [M-3H]⁻³. Aldehyde1_antizip2 reporter1 (DNA: SEQ ID NO: 69): yield 40 %. Calcd for $C_{305}H_{398}N_{110}O_{177}P_{29}$ (monoisotopic): 1331.96239 [M-7H]⁻⁷; Found: 1332.0778 [M-7H]⁻⁷.

[0129] Example 5 to 8 are related to the preparation of indole and indolinium analogues for DNA conjugation.

[0130] Indole analogues can be synthesized following the general Fischer-indole synthesis by converting aryl hydrazones to indoles under acidic conditions (Scheme 7). First, a primary aromatic amine and nitrous acid reacts to give a diazonium salt. The diazonium salt is then reduced to a hydrazine (Hunsberger *et. al. J. Org. Chem.* **1956**, 21, 394–399). Finally hydrazine reacts with 3-methylbutan-2-one to form the aryl hydrazone which upon isomerization and elimination of NH₃ forms indole (Lindsey *et. al. Tetrahedron* **1989**, 45, 4845–4866).

Scheme 7: General synthetic routes to the indole and indolinium analogues.



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Example 5: Synthesis of 5-methoxy-2,3,3-trimethyl-3H-indole

[0131] To a solution of 4-methoxyaniline (2.46 g, 20 mmol) in 60 mL of conc. HCl was added dropwise the solution of NaNO₂ (1.38 g, 20 mmol) in 35 mL of H₂O at 0 °C. After stirring for 0.5 h at 0 °C, the reaction mixture was added dropwise to a solution of SnCl₂ (9.03 g, 40 mmol) in 35 mL of conc. HCl at 0 °C, then stirring was continued for 1.5 h at 0 °C. 2N NaOH was then added to quench the reaction until pH = 9 to 10. The aqueous layer was extracted with DCM (50 mL x 3) and the organic layer was dried over Na₂SO₄. After filtration and concentration, the desired product, (4-methoxyphenyl)hydrazine was obtained (0.98 g, 35 % yield), which was used directly for next step.

[0132] A mixture of (4-methoxyphenyl)hydrazine (0.98 g, 7.1 mmol) and 3-methylbutan-2-one (1.53 g, 17.8 mmol) in 20 mL of HOAc was heated at 100 °C overnight. The mixture was concentrated and 1N NaOH was added until pH 9 to 10. The aqueous solution was extracted with ethyl acetate (50 mL x 3). The combined organic layers were dried over Na₂SO₄. After filtration and concentration, the residue was purified by flash column chromatography to give 610 mg of 5-methoxy-2,3,3-trimethyl-3H-indole (45 %). ¹H NMR (CDCl₃) δ_{ppm}: 7.43 (dd, 1H), 6.81 (m, 2H), 3.83 (s, 3H), 2.26 (s, 3H), 1.28 (s, 6H). LC-MS (M+H): 190.16

Example 6: Synthesis of 4(6)-nitro-2,3,3-trimethyl-3H-indole

[0133] (3-nitrophenyl)hydrazine (MW=189.6, 685 mg, 3.6 mmol) and 0.75 ml 3-methyl-2-butanone were stirred in 8 mL of EtOH at RT for 10 minutes, then at 40 °C for 15 minutes. Ethanol was removed under reduced pressure. The residue was taken up in 20 mL of conc. HCl and heated at 100 °C for 2h. The aq. HCl was then removed under reduced pressure. The solid was triturated with 2 mL of iced water, filtered and washed with 2 mL of iced water. After drying in the air, the solid weighed 300 mg. However, TLC indicated that it contained a mixture of two products. The aqueous layers were neutralized with 1N NaOH to pH~8.0, extracted with EtOAc (50 mL x 3). The organic layers and the solid obtained previously were combined, dried over Na₂SO₄ overnight. The organic solution was filtered, washed with EtOAc, and concentrated under reduced pressure. The residue was purified by flash chromatography (SiO₂, 70 g), eluted with 8-15% EtOAc in hexane to give two fractions. The first fraction was obtained in 217 mg (30% yield) as a light yellow oil, which was identified as compound 4-nitro-2,3,3-trimethyl-3H-indole by ¹H NMR. ¹H NMR (CDCl₃) δ_{ppm}: 8.0 (dd,

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1H), 7.89 (dd, 1H), 7.50 (t, 1H), 2.73 (s, 3H), 1.50 (s, 6H). The second fraction was obtained in 245 mg (33% yield) as a yellow solid, which was identified as 6-nitro-2,3,3-trimethyl-3H-indole by ¹H NMR. ¹H NMR (CDCl₃) δ_{ppm}: 8.33 (d, 1H), 8.12 (dd, 1H), 7.50 (t, 1H), 7.38 (d, 1H), 2.33 (s, 3H), 1.27 (s, 6H).

Example 7: Synthesis of 2,3,3-trimethyl-3H-benzo[g]indole-5-sulfonic acid.

[0134] A solution of sodium 4-aminonaphthalene-1-sulfonate (2.45 g, 10 mmol) in H₂O (15 mL) was added a solution of NaNO₂ (0.70 g, 10 mmol) in H₂O (2 mL) at 10-15 °C. The solution was then added to a cold solution of conc. H₂SO₄ (0.54 g, 5.5 mmol) in 0.5 mL of H₂O. The temperature was maintained below 10 °C and the mixture was stirred for 1.5 h after the addition was complete. The mixture was then added dropwise to a cold solution of SnCl₂ (3.8g, 17 mmol) in 2.5 mL of conc. HCl and 1.5 ml of H₂O. The reaction temperature was kept below 10 °C and allowed to sit overnight. It was filtered and washed thoroughly with H₂O. The cake was removed twice from the Buchner funnel and suspended in H₂O and filtered. The solid obtained was dried under vacuum to give 1.4 g of 4-hydrazinylnaphthalene-1-sulfonic acid (59%). The material was used directly for next step. A solution of 4-hydrazinylnaphthalene-1-sulfonic acid (0.7g, 2.9 mmol) in 3 ml of HOAc was added 3-methylbutan-2-one (0.5 ml, 1.5 eq), NaOAc (0.47g, 2.0 eq.). The mixture was stirred at 110 °C for 3.5h. After cooling and addition of ether, the precipitate was filtered to give 700 mg. The solid was dissolved in DCM and purified by flash chromatography on SiO₂ using 10% MeOH in DCM to give 273 mg of product. The mother liquor was concentrated and was also purified by flash chromatography on silica gel to give additional 372 mg of product. The overall yield was 77%. ¹H NMR (DMSO) δ_{ppm}: 8.8 (dd, 1H), 8.4 (dd, 1H), 8.0 (s, 1H), 7.5 (m, 2H), 3.1 (s, 5H), 2.3 (s, 4H), 1.3 (s, 6H). LC-MS: 288.2 [M-H].

Example 8: Synthesis of 3-(4-carboxybutyl)-1,1,2-trimethyl-1H-benzo[e]indolium-7-sulfonate.

[0135] To a solution of 6-aminonaphthalene-2-sulfonic acid (2.23g, 10 mmol) in 15 ml of H₂O was added 2N NaOH (0.75 ml). The mixture was stirred for 5 min at RT and conc. H₂SO₄ (0.68 g, 6.9 mmol) was added to it dropwise at 0 °C. 2 ml of iced H₂O was added, followed by a solution of NaNO₂ (1.04 g, 15 mmol) in 2 ml of H₂O. After the solution was stirred at 0 °C for 2h, the diazonium salt was removed by filtration and washed with cold water. The salt was

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then added in portions to a cold (<0 °C) solution of SnCl₂ (4.9g, 22 mmol) in 3.2 ml of conc. HCl and 1.8 ml of H₂O. The mixture was stirred overnight. The solid was filtered and washed twice with water, dried under vacuum to give 1.84 g of 6-hydrazinylnaphthalene-2-sulfonic acid (77%), which was used directly for next step.

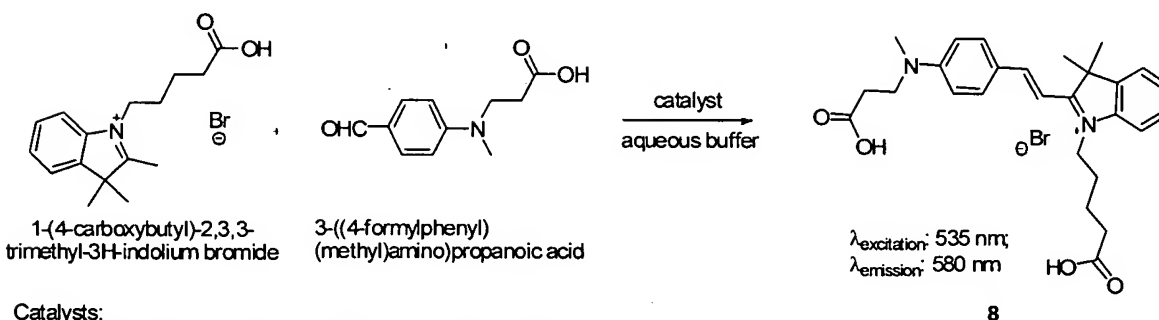
5 [0136] To a solution of 6-hydrazinylnaphthalene-2-sulfonic acid (0.7 g, 2.9 mmol) in 3 ml of HOAc was added 3-methylbutan-2-one (0.5 ml, 1.5 eq), NaOAc (0.47g, 2.0 eq.). The mixture was stirred at 110 °C for 3.5h. After cooling, the solvent was removed under reduced pressure. The residue was dissolved in MeOH and 3.0g of SiO₂ was added. MeOH was removed and the silica gel was loaded on a silica gel column and eluted with 10% MeOH in DCM to give the
10 desired product, 1,1,2-trimethyl-1H-benzo[e]indole-7-sulfonic acid (874 mg, >95% yield). LC-MS: 288.2 [M-H]

[0137] To a solution of 1, 1, 2-trimethyl-1H-benzo[e]indole-7-sulfonic acid (723mg, 2.5 mmol) in methanol (5 mL) was added a saturated solution of potassium hydroxide in isopropanol (3.134%, 4.913g, 1.1 eq.) and the resulting suspension was heated at reflux for 2h.
15 Then the mixture was concentrated and the corresponding potassium salt was obtained. Under nitrogen atmosphere, a mixture of potassium 1, 1, 2-trimethyl-1H-benzo[e]indole-7-sulfonate and 5- bromovaleric acid (585mg, 3 mmol) in 3-methyl-2-butanone (5 mL) was heated at 140 °C for 20h. Removal of the solvent and purification by chromatography (dichloromethane / methanol) afforded 3-(4-carboxybutyl)-1,1,2-trimethyl-1H-benzo[e]indolium-7-sulfonate (70
20 mg, isolated yield 7.2%). LC-MS: 390.18 [M+].

Example 9 Organocatalyzed hemicyanine synthesis in aqueous buffer.

[0138] **Scheme 8** provides an example of synthesizing hemicyanine **8** in aqueous buffer in the presence of various catalysts. The extent of hemicyanine formation was easily monitored by analytical reversed-phase HPLC (UV at 545 nm). Hemicyanine **8** has fluorescence excitation
25 wavelength maximum at 535 nm and emission maximum at 580 nm. MALDI-MS analysis of the product confirms the structure (M⁺: 449.1992). The experimental data indicates (S)-pyrrolidinemethylpyrrolidine ((S)-PMP) has better catalytical ability than other catalysts.

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Scheme 8: Hemicyanine formation in aqueous buffer in the presence of catalysts

Catalysts:

- (1) L-valine (0.3 equiv. water); (2) L-proline (0.3 equiv. water);
 (3) (S)-Pyrrolidinemethylpyrrolidine (0.3 equiv. 50 mM sodium phosphate buffer; pH 8.5);
 (4) (S)-2-pyrrolidinemethanol (0.3 equiv. water); (5) Zinc-proline or Zn(Pro)₂ (0.1 equiv. 20 M sodium phosphate buffer, pH 8.5);
 (6) Pyrrolidine/acetic acid (0.4/0.2 equiv. water); (7) Pyrrolidine/10-camphorsulfonic acid (0.4/0.2, water)

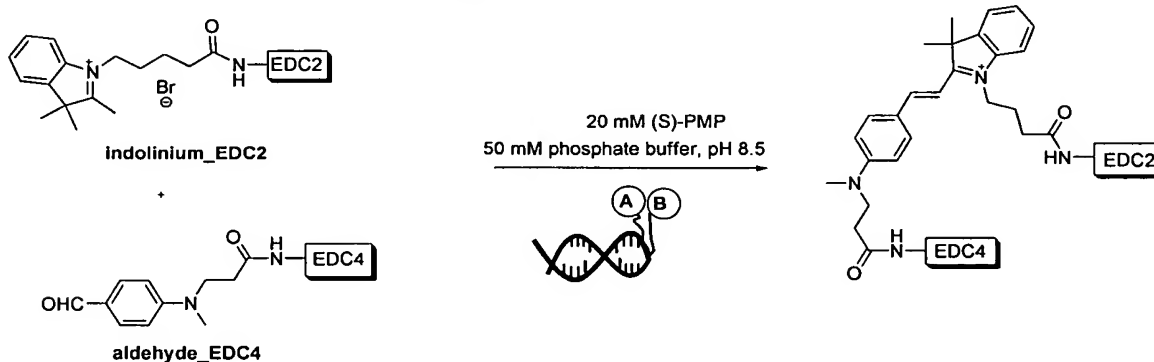
Reaction conditions: 25 mM each of the starting material, 20% NMP in water or sodium phosphate buffer plus required catalysts, RT for 16 hr.

Examples 10-13 are related to DPC of hemicyanine formation.

5 Example 10 DPC hemicyanine formation (end of helix architect)

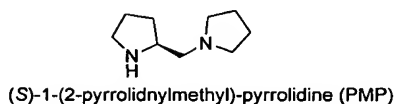
[0139] **Scheme 9** gives an example of DPC hemicyanine formation through end of helix architecture. Upon annealing, the two hemicyanine precursors were placed in reactive proximity at the end of helix and a hemicyanine linked to both DNA was formed after condensation.

10 Scheme 9: Example of synthesizing hemicyanine_DNA dye 1 through DPC (end of helix).



Name Sequence (5' - 3')
 EDC2 H2N-AGCTC CAACT ACCAC
 EDC4 GTGGT AGTTG GAGCT-NH2
 EDC3 H2N-AGATC CCACT AGCAC (mismatch)

(SEQ ID NOS: 75-77)



[0140] The progress of DPC reaction was monitored by fluorescence spectroscopy. FIG. 17 shows the fluorescence emission of DPC reaction mixture of indolinium and aldehyde DNA (I_EDC2 and A_EDC4) (DNA: SEQ ID NO: 75; SEQ ID NO: 77) at various conditions. First, there is no background fluorescence emission for hemicyanine precursor I_EDC2 (DNA: SEQ ID NO: 75) and A_EDC4 alone in the reaction buffer (2 & 3 in FIG. 17). Second, the DPC reaction is catalyst dependent and not pH dependent. Without the addition of (S)-PMP, there is no fluorescence signal. Simply increasing the pH of the reaction conditions from pH 8.4 to 10.0 did not generate any signal (5 & 6 in FIG. 17). Finally, DPC reaction is Watson-Crick sequence-specific (nucleic acid dependent). By switching EDC4 with three mismatched nucleobases DNA (EDC3, DNA: SEQ ID NO: 76), no fluorescence signal was generated after 140 minutes. Only trace amount of fluorescence signal detected after 16 hr (FIG. 18). After 16 hr, LC-MS shows almost 90% conversion of the product for reaction 1 (FIG. 19). FIG. 20 gives the electrospray mass data of purified DPC product (EDC4_H_EDC2) (DNA: SEQ ID NO: 77; SEQ ID NO: 75) which confirms the structure. The relative quantum yield measured for EDC2_H_EDC4 (DNA: SEQ ID NO: 75; SEQ ID NO: 77) is around 0.1 in water using sulforhodamine 101 as fluorescence standard. The extinction coefficient of EDC2_H_EDC4 (DNA: SEQ ID NO: 75; SEQ ID NO: 77) at 550 nm is around 87000 in water.

[0141] DPC reaction: Reactions were performed with 200 nM each of reagent in 10 mM (S)-PMP, 50 mM sodium phosphate buffer, pH 8.4, 1 M NaCl at RT unless otherwise specified.

Catalyst (S)-PMP was added after mixing both reagents together in reaction buffer.

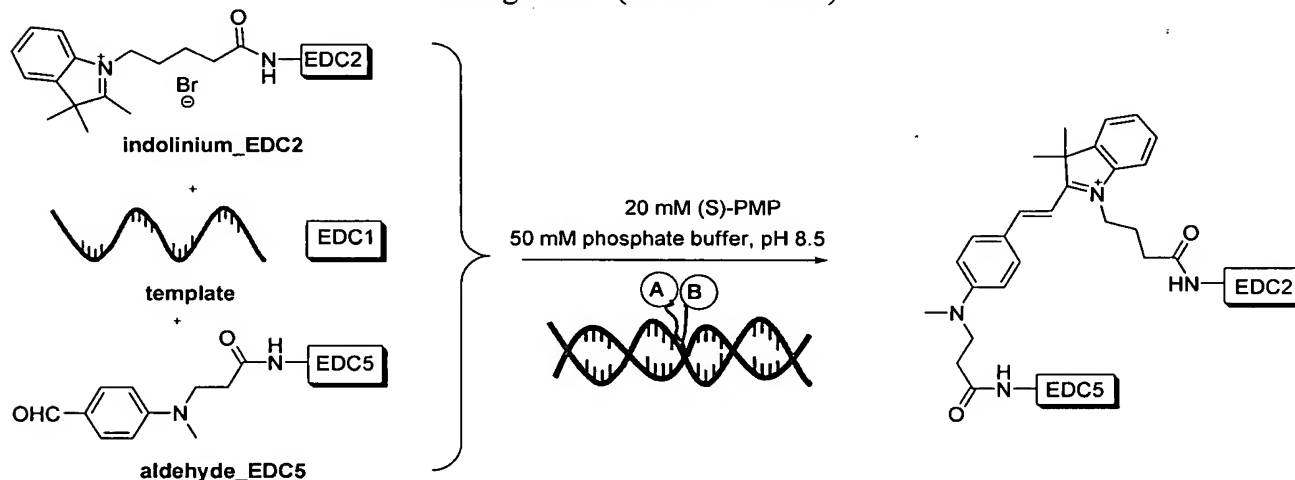
Example 11 DPC hemicyanine formation (middle of helix architect)

[0142] Scheme 10 provides another example of DPC hemicyanine formation through middle of helix architecture where the reactants were labeled to two probes which can complementary with a single template. Upon annealing, the two hemicyanine precursors were placed in reactive proximity at the middle of helix and a hemicyanine linked to both DNA was formed. The experimental data indicate only in the presence of the template, fluorescence signal is generated (FIG. 21). EDC2_H_EDC5 (DNA: SEQ ID NO: 75; SEQ ID NO: 78) was purified and its structure was confirmed by mass data (FIG. 22). The relative quantum yield measured for EDC2_H_EDC5 (DNA: SEQ ID NO: 75; SEQ ID NO: 78) alone is similar to EDC2_H_EDC4 (DNA: SEQ ID NO: 75; SEQ ID NO: 77) (0.1 in water). The extinction

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coefficient of EDC2_H_EDC5 (DNA: SEQ ID NO: 75; SEQ ID NO: 78) at 550 nm is around 75000 in water.

Scheme 10: Example of synthesizing hemicyanine_DNA dye 2 through DPC (middle of helix).



Name Sequence (5' – 3')
 EDC1 GTGGT AGTTG GAGCT GGTGG CGTAG GCAAG A
 EDC2 H2N-AGCTC CAACT ACCAC
 EDC5 TCTTG CCTAC GCCAC-NH2
 (SEQ ID NOs: 74, 75, and 78)

[0143] DPC reaction: Reactions were performed with 200 nM each of indolinium_EDC5 (DNA: SEQ ID NO: 78), aldehyde_EDC5 (DNA: SEQ ID NO: 78), EDC1 (DNA: SEQ ID NO: 74) in 10 mM (R)-PMP, 50 mM sodium phosphate buffer, pH 8.4, 1M NaCl at RT. (S)-PMP was added after mixing both reagents and template together in the reaction buffer.

Example 12 Kinetic studies of hemicyanine DPC

[0144] The reaction rate for both end-of-helix (Example 10) and middle-of-helix (Example 11) DPC were investigated by fluorescence spectroscopy (FIG. 23 and FIG. 24). The kinetic profiles for both reactions are very similar. After around 16 hr, the fluorescence signal reached plateau which indicated the completion of the reaction.

Example 13 Generation of four fluorescent hemicyanine dyes via middle-of-helix DPC.

[0145] FIG. 3 gives an example of four-plex hemicyanine_DNA dyes that can be generated through DPC. In this example, total two quaternary salt precursors (indolinium I and benzoindolinium BI) and two aldehydes (A0, A1) are needed to generate four DNA hemicyanine dyes. These precursors share the structural similarity, thus DPC conditions for

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quaternary salt and aldehyde should be similar. According to literature data, benzoindolinium compound generally gives 20 nm of red shift while one extra double bond conjugation shifts the fluorescence emission wavelength towards to visible range (~ 80 nm for hemicyanine dye). **FIG. 3** lists the spectroscopic properties of these four hemicyanine_DNA dyes.

5 **[0146]** Small molecule hemicyanine dyes (**9** to **12**) were first synthesized and their fluorescence excitation and emission spectra were recorded (**FIG. 25**). As predicted, one extra phenyl substitution shifted the fluorescence emission 20 nm to the red (compound **9** and **10**), while each additional vinyl group in the polyene chain shifted around 80 nm (**9** and **11**; **10** and **12**). To maximize the utility of the DNA codons and simplify the DNA probe preparation, only
10 two DNA strands was used to label **I** and **BI** and two DNA strands for aldehydes with four template strands (**FIG. 26**). Each template had two unique codons that can only Watson-Crick base pair with one set of aldehyde and quaternary salt precursor to generate one hemicyanine dye. The DPC was performed in a combinatorial fashion in one-pot. **FIG. 27** shows normalized fluorescence emission spectra of four individual DPC reactions between indolinium/aldehyde
15 (a) and benzoindolinium /aldehyde (b), indolinium/ α,β -unsaturated aldehyde 1 (c) and benzoindolinium/ α,β -unsaturated aldehyde 1 (d) using above mentioned DNA codon (**FIG. 26**). Four colors were generated from these DPC reactions. Similar to the fluorescence data from small molecule hemicyanine dyes (**FIG. 25**), around 15 nm of fluorescence emission wavelength difference was observed for one extra phenyl substitution (compound **13** and **14**, **15**
20 and **16**). One additional vinyl group in the polyene chain shifted around 80 nm of fluorescence emission wavelength (compound **13** and **15**, **14** and **16**).

[0147] DPC reaction: Reactions were performed with 200 nM each of strands and template in 10 mM N,N-dimethyl ethylenediamine (DMEDA), 50 mM sodium phosphate buffer, pH 8.4, 150 mM NaCl at RT. Catalyst was added after mixing both reagents and template together in
25 the reaction buffer.

Example 15. Generation of two fluorescent hemicyanine dyes via end of helix DPC

[0148] Two hemicyanine products were formed by mixing antizip3_indolinium with antizip2 reporter1_A0 and antizip2 reporter1_A1 respectively (DNA: SEQ ID NO: 69). The product (**17**) formed between antizip3_indolinium (DNA: SEQ ID NO: 70) and antizip2 reporter1_A0
30 (DNA: SEQ ID NO: 69) has excitation maximum at 540 nm and emission maximum at 600

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nm, while the product (18) formed between antizip3_indolinium (DNA: SEQ ID NO: 70) and antizip2 reporter1_A1 (DNA: SEQ ID NO: 69) had excitation maximum at 600 nm and emission maximum at 670 nm (FIG. 28).

[0149] DPC reaction: Reactions were performed with 200 nM each of reagent in 15 mM DMEDA, 50 mM sodium phosphate buffer, pH 8.0, 2.5 mM MgCl₂ at 30 °C. Total reaction volume was 50 µL. Catalyst DMEDA was added after mixing both reagents together in reaction buffer. Fluorescence was recorded immediately after the addition of catalyst DMEDA.

GENERAL EXAMPLES ON DPC-BASED PROTEIN DETECTION

Example 16. Creation of Fluorescence by Hybridization Induced Azidocoumarin

Reduction

[0150] Five oligonucleotides were prepared using standard phosphoramidite chemistry (Glen Research, Sterling VA, USA). Oligonucleotides bearing 5'-amino groups (Oligo2 and Oligo6) were prepared using 5'-Amino-Modifier 5 and Oligonucleotides bearing 3'-aminogroups (Oligo4 and Oligo5) were prepared using 3'-Amino-Modifier C7 CPG (Glen Research, Sterling VA, USA)

Oligo1	5'-GTGGTAGTTGGAGCTGGTGGCGTAGGCAAGA-3'	(SEQ. ID. NO. 19)
Oligo2	5'-H2N-AGCTCCAACTACCAC-3'	(SEQ. ID. NO. 20)
Oligo4	5'-GTGGTAGTTGGAGCT-NH2-3'	(SEQ. ID. NO. 21)
Oligo5	5'-TCTTGCCTACGCCAC-NH2-3'	(SEQ. ID. NO. 22)
Oligo6	5'-H2N-AGATCCCACTAGCAC-3'	(SEQ. ID. NO. 23)

[0151] Oligo1, Oligo4 and Oligo5 were removed from the synthesis support and purified by reversed-phase HPLC. The amino groups of Oligo2 and Oligo6 were converted while resin-bound to their triphenyl phosphine derivatives and these were purified and isolated (Sakurai *et al.*, J. Amer. Chem. Soc., 2005, 127, pp1660-1667) to give Oligo2-TPP and Oligo-6TPP, respectively.

[0152] Amino group bearing Oligo4 and Oligo5 were converted to their azidocoumarin derivatives (Oligo4-AzC and Oligo5-AzC, respectively) by reaction of each oligo with the N-hydroxysuccinimide ester of 7-azido-4-methylcoumarin-3-acetic acid (Thevenin *et al.*, Eur. J. Biochem (1992) Vol. 206, pp-471-477). The reaction was performed by adding 1 µL of trifluoroacetic acid to 5 µL of N-methylmorpholine to prepare a buffer to which was added 10 µL of water containing 6.6 nmol of Oligo 4 or Oligo 5, followed by addition of 30 µL of a 0.16

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M solution of the coumarin NHS-ester in dimethylformamide. Each reaction was allowed to proceed for 2 hours at room temperature, whereupon 50 μ L of 0.1 M aqueous triethylammonium acetate was added. The mixtures were applied to a NAP-5 desalting columns (Amersham Biosciences, Piscataway NJ USA) and eluted according to the manufacturers instructions the eluate was purified by RP-HPLC to provide Oligo4-AzC and Oligo5-AzC, in yields of 77% and 70%, respectively. Product identity was confirmed by Maldi-ToF mass spectrometry.

[0153] To demonstrate the hybridization-specific creation of fluorescence, various combinations of complementary and non-complementary oligonucleotides bearing azido-coumarin and triphenyl phosphine moieties were allowed to react at room temperature in a buffer comprised of 30% aqueous formamide, 50 mM NaCl, and 10 mM sodium phosphate, pH 7.2. The reaction progress was monitored over time using a Victor Multilabel fluorimeter (EG&G Wallach, Turku Finland) set to excite the sample at 360 nm and monitor light emission at 455 nm

[0154] FIG. 29 shows that when Oligo4-AzC and Oligo2-TPP are combined to final concentrations of 200 nM and 400 nM respectively, a rapid increase in fluorescence is observed. In this figure 004 denotes Oligo4-AzC, 002 denote Oligo2-TPP, and 006 denotes Oligo6-TPP. The fluorescence does not occur when Oligo6-TPP is substituted for Oligo2-TPP. Whereas Oligo2-TPP is perfectly complementary in its base-pairing ability to Oligo4-AzC, Oligo6-TPP is not, as it contains three mismatched nucleotides. The results support the conclusion that the creation of fluorescence is due to the ability of Oligo2-TPP to hybridize to Oligo4-AzC thus facilitating a reaction between the TPP and azidocoumarin moieties in the resulting hybrid. The lack of signal in the case of reaction of Oligo6-TPP with Oligo4-AzC is consistent with inability of these two oligonucleotides to form a duplex, therefore the reaction is not facilitated. Control reactions containing each single oligonucleotide were performed to rule out any non-specific effects.

[0155] Results of additional experiments involving ternary complexes are shown in FIG. 30. In these experiments Oligo1 is tested for its ability to bring together by hybridization two perfectly complementary oligonucleotides (Oligo5-AzC and Oligo-2TPP) versus its ability to bring together one perfectly complementary oligonucleotide (Oligo5-AzC) and one partially-complementary oligonucleotide (Oligo6-TPP). Oligo1 and Oligo5-AzC were at 200 nM final

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concentration, whereas Oligo2-TPP and Oligo6-TPP were employed at 400nM final concentration. In **FIG. 30**, 001 denotes Oligo1, 002 denotes Oligo2-TPP, 005 denotes Oligo5-AzC, and 006 denotes Oligo6-TPP. The results show that fluorescence is generated only when the combination of fully complementary oligonucleotides is present (Oligo1, Oligo5-AzC and Oligo2-TPP).

Example 17. Oligonucleotide Hybridization, Concentration and Melting Temperatures

[0156] A model system was prepared which included two twenty-mer oligonucleotides with a ten-base complementary region and ten-base single stranded spacer arms, further linked to a six carbon spacer arm. These oligos were synthesized both with and without a 5'-biotin (with a 6-carbon spacer arm). As shown below, the complementary region is underlined. A third oligo was identical to the (-) strand oligo but with 4 base mismatches (*italicized*) to the (+) strand.

Oligo 26	(+) strand	5'	CTTCGGCCCAGATATCGT	(SEQ. ID. NO. 24)
Oligo 27	(-) strand	3'	<u>GTCTATAGCATCGACATC</u>	(SEQ. ID. NO. 25)
Oligo 28	(-) mismatch	3'	<u>TACTATAGTGT</u> CGACATC	(SEQ. ID. NO. 26)

[0157] Melting curves of the 10-base pair oligonucleotide pair (oligo 26+oligo 27) were examined by measuring fluorescence of SYBR dye binding to double stranded DNA in a Bio-Rad iCycler (Lipsky, *et al.*, Clinical Chemistry **2001**, 47[4], 635-44). The binding curves are presented as the first derivative of the slope of the melting curve, such that a maximum value represents a point of inflection in the curve (a T_m , or in a mixed population of double stranded sites, a "local" T_m). Binding curves can be obtained up to at least 70° C as avidin retains biotin binding activity up to this temperature and beyond.

[0158] To check the dependence of this particular pair of oligonucleotides upon concentration, melting curves were generated for the oligonucleotide pair varied over the range from 500 to 20 nM (**FIG. 31**). (See, e.g., Lipsky, *et al.*, Clinical Chemistry **2001**, 47[4], 635-44). The observed T_m dropped at the rate of about 10°C per each ten-fold reduction in concentration (where RFU indicates relative fluorescence units) of the oligonucleotide pair, similar to prediction in the graph of **FIG. 31**. The melting curves were essentially identical for biotinylated and non biotinylated oligonucleotide pairs. The four base mismatched pair showed essentially no double stranded structure.

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[0159] To test whether binding the (+) and (-) strands to a protein target would cause an increase in T_m , the biotinylated version of these oligonucleotides were incubated in the presence of avidin. Avidin contains 4 equivalent binding sites, which are spaced relatively close together and bind to biotin very tightly ($K_a \sim < 10^{-15}$ M) and non-cooperatively.

5 [0160] Presented with equal molar concentrations of oligonucleotides #26 and #27 in biotinylated form, it would be expected that about half of the biotin binding sites are occupied by complementary pairs of oligonucleotides, and about half with the same oligonucleotide (non-complementary pairs). The prediction is that one would observe two melting curve peaks in the presence of avidin. One peak would be the result of any pairs of oligonucleotides which
10 were either not bound to avidin (free in solution) or which had only one partner of the two bound to avidin, which should not exhibit a proximity effect upon T_m . A second peak of significantly higher T_m would represent a pair of biotinylated oligos both bound to avidin, which should exhibit a proximity effect.

[0161] Such an experiment was conducted as shown in FIG. 32. The oligonucleotides were
15 added to a solution in the presence or absence of avidin held at 60° C, a so-called hot start. In a "hot start," the oligonucleotides bind to the biotin binding sites at a temperature well above their T_m in solution, assuring that they are single stranded. The solution was then ramped down to 10° C and a melting curve analysis performed ascending to 70° C. As shown in FIG. 32, the melting curves of non-biotinylated oligo pair in the presence or absence of avidin showed a T_m
20 of 30-32° C (where RFU indicates relative fluorescence units). In the presence of avidin, however, two well separated T_m peaks were generated with T_m values of 33° C and 52° C. The elevated temperature peak (T_m raised almost 20° C) was observed only in the presence of two complementary biotinylated oligonucleotides in the presence of avidin. The difference in T_m +/- biotin tended to be greatest at lower salt concentrations (FIG. 33) and slightly higher in the
25 presence of 10 mM magnesium chloride (FIG. 34) (where RFU indicates relative fluorescence units). The optimal molar ratio of biotinylated oligonucleotides to avidin was found to be about 3.5:1, (with total concentration of oligos + avidin = 0.7 μ M) consistent with avidin possessing four equivalent binding sites (FIG. 35) (RFU indicates relative fluorescence units). This is important because it substantiates that the requirement that the oligonucleotides bind to the
30 same molecule of avidin for the T_m effect to occur. The substitution of a 3' biotinylated (-) strand oligo for a 5' biotinylated strand oligonucleotide showed little difference in T_m values

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(**FIG. 36**) (RFU indicates relative fluorescence units) with previous results in which both oligonucleotides were 5' biotinylated.

[0162] Results were essentially identical if the experiment was conducted by adding equimolar amounts of both the oligonucleotides at room temperature, ramping to 60° C, and then obtaining the melting curves. In this method (as well as the hot start method) suitable melting curves can be generated by adding an excess molar of each oligo relative to avidin if desired. (Large excesses of pairs of oligos increases the size of the low T_m peak, however, as predicted.) This was not detrimental in forming high T_m hybrid DNA since the pairs of oligos competed equally for biotin binding sites as long as they were added together in equal molar amounts. If oligos were added one at a time, it was important to add about a 2:1 molar ratio of the first oligo to avidin followed by a 2:1 ratio of the second oligo. With sequential addition, adding an excess molar amount of either oligo relative to avidin occupies all the binding sites of the avidin with the first oligo and prevents occupying adjacent sites with the second, complementary oligo and exhibiting the elevated T_m effect. These observations are consistent with the mechanism being binding of adjacent pairs of complementary oligos to two adjacent biotin binding sites to obtain hybrids exhibiting the elevated T_m peaks.

[0163] Experiments were also conducted with a 10-base self-complementary oligonucleotide which was composed entirely of A and T. (Oligo 31: 5'-biotin-spacer arm-TTTTTTTTTTTTAAATTAATAA) (SEQ. ID. NO. 27). Because this oligonucleotide was homogeneous in base composition and composed entirely of AT, it melted at a lower T_m than the above-described model system and produced a fairly sharp melting curve. In the presence of avidin, its T_m was increased from 30.5° C to 61.5° C (**FIG. 37**) (where RFU indicates relative fluorescence units). Since this oligonucleotide was self-complementary, all binding events lead to complementary strands, rather than only ½ of the events. Thus, only a single peak of increased T_m was observed.

[0164] These experiments were repeated using anti-biotin antibody as a target rather than avidin. Anti-biotin antibody contains two biotin binding sites located near the ends of the Fab portion of the antibody, but the binding sites are much further apart than the biotin binding sites on avidin.

Example 18. Detection of Protein Targets – Aptamers as Target Binders

[0165] Here, an exemplary system was designed to utilize nucleic acid-templated azidocoumarin (AzC) –triphenylphosphine (TPP) chemistry to detect a protein target upon aptamer binding and annealing of the two complementary DNA probes.

5 **Materials**

[0166] Human PDGF-BB and PDGF-AA was obtained from R&D Systems (220-BB and 220-AA, respectively). Anti-human PDGF-B Subunit monoclonal antibody was obtained from R&D Systems (MAB2201). Buffers included Tris/Mg buffer, at 50 mM Tris/HCl, pH 8.0 – 10 mM MgCl₂. Oligonucleotides used were as follows:

Oligonucleotide Sequences Used in this Example

Oligo #/ (SEQ. ID #)	Sequence (5' to 3')	5' – Mod' f.	3'- Mod 'f.	Description
201 (28)	<u>CAGGCTACGGCACGTAGAGCATCACCATG</u> <u>ATCCTGCCCCCCCCCATATTTAAGC</u>	TPP	none	DPC-aptamer probe
202 (29)	GCTTAAATATCCCCCCCCC <u>CAGGCTACGG</u> <u>CACGTAGAGCATCACCATGATCCTG</u>	none	AZC	DPC-aptamer probe
203 (30)	GTGGGAATGGTGCCCCCCCCC <u>CAGGCTAC</u> <u>GGCACGTAGAGCATCACCATGATCCTG</u>	none	AZC	DPC-aptamer probe-mismatch
204 (31)	GTGGTAGTTGGAGTCGTGGCGTAGGCAAG A	none	none	target
205 (32)	GTGGTAGTTGGAGTCACACGTGGCGTAGG CAAGA	none	none	target
206 (33)	GTGGTAGTTGGAGCTCACACCACACGTGGC GTAGGCAAGA	none	none	target
207 (34)	GTGGTAGTTGGAGTCACACACACCACACA CAGTGGCGTAGGCAAGA	none	none	target
208 (35)	GTGGTAGTTGGAGCTCACACCACACCAACC ACACCACACCACACACACCACACGTGGCG TAGGCAAGA	none	none	target
209 (36)	GTGTGGTGTGGTGTGGTGTG	none	none	splint
210 (37)	GTGGCGTAGGCAAGAGTGGTAGTTGGAGC T	none	none	K-ras target outward facing
211 (38)	GTGGGAATGGTG	none	TPP	TPP probe

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212				
(39)	AGATCCCACTAGCAC	TPP	none	TPP probe
213				
(40)	AGCTCCAACCTACCAC	TPP	none	TPP "mismatch"
214				
(41)	TCTTGCCTACGCCAC	none	AZC	AZC probe
215	CAGGCTACGGCACGTAGAGCATCACCATG			
(42)	ATCCTG	none	none	aptamer

Methods

[0167] *DPC Reaction conditions.* Except as noted, each 100 microliter reaction contained, in a total volume of 100 μ l, 1 xTris/Mg buffer, 40 picomoles of TPP and AzC reaction probes, 40 picomoles of target oligonucleotide or of target protein, and typically 25-30% v/v of formamide. Samples were incubated at 25° C in a Wallac Victor 1420 spectrophotometer and the increase in fluorescence monitored with excitation at 355 nm and emission at 460 nm.

Results: Detection of PDGF-BB by Aptamer-DPC Probes

[0168] As illustrated in **FIG. 38**, an aptamer sequence directed against platelet-derived growth factor (PDGF) B-subunits was selected (Fang, *et al.*, Chem. BioChem. **2003**, 4, 829-34.). This belongs to a family of aptamers with strong affinity for PDGF B subunit ($\sim 10^{-9}$ M), and about ten-fold reduced affinity for PDGF A subunit. (Green, *et al.*, Biochemistry 35, 14413-24. 1996) The probe sequences were synthesized, each containing a complementary 10-mer DNA sequence, a C₁₀ spacer sequence, and the same 35-mer aptamer sequence. (Oligos #201, #202). Each sequence contained a 5'-TPP or 3'-AZC group with the aptamer linked 3' or 5', respectively. A second AzC probe, oligo #203, was the same as oligo #202 except that its annealing sequence was entirely mismatched to the TPP oligo (#201).

[0169] As shown in **FIG. 39**, in the presence of 30% (volume) formamide, the reaction of the TPP and AzC probes with each other was entirely dependent upon the presence of PDGF-BB and complementary DNA sequences on the probes. The reaction failed in the absence of either probe.

[0170] The DNA-dependence of the reaction was critically dependent upon the melting temperature of the DNA relative to the assay temperature. In the presence of 0% formamide (with the calculated and observed $T_m > T_{\text{assay}}$, the reaction took place in the presence or absence of the target protein PDGF-BB (**FIG. 40A**). In fact, under these conditions, addition of PDGF-BB did not increase, but reduced the reaction rate by about 50%. In 10% formamide, PDGF-

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BB was less inhibitory (**FIG. 40B**). In 20% formamide (**FIG. 41A**), the situation was completely reversed – the reaction rate was now weak except in the presence of PDGF-BB. In 30% formamide (**FIG. 41B**) the reaction was completely dependent upon the presence of PDGF-BB. In 40% formamide, the reaction was very slow with any set of reactants (**FIG. 42**).

5 In all cases, the mismatched probes produced little or no reaction.

[0171] DNA melting experiments with the complementary sequences, monitored with SYBR Green had indicated a T_m of the sequence of about 30° C in the Tris/Mg buffer in the absence of formamide, and about 7° C lower for every 10% increase in formamide. T_m in the optimal formamide concentration for the detection assay, 30%, was 10° C.

10 [0172] In 0% formamide, the oligonucleotides can form at least a partial duplex even in the absence of PDGF-BB (T_m slightly higher than T_{assay}). The DNA target-dependence of the reactions in 20% and 30% formamide is explained by the assay being conducted at a temperature greater than the T_m in the absence of protein target. No reaction occurs unless the T_m of the complex is increased by the binding of the two probes to the PDGF-BB target. At
15 40% formamide, the reaction doesn't occur with any set of reactions. The likely explanation is that either the T_m had been reduced so low that binding to PDGF-BB could not raise it above T_{assay} , or that formamide had inhibited PDGF-BB binding to the aptamers. A more complex situation is the observed inhibition of reaction rate upon addition of PDGF-BB in the absence of formamide. Since half of the duplexes formed by PDGF-BB are non-productive (50% will be
20 homoduplexes) the reduction in rate is likely due to PDGF-BB binding preventing these homoduplexes from disassociating and then reassociating in solution with complementary pairs to form heteroduplexes. This situation should not occur using pairs of probes specifically directed against different binding sites in a heterodimeric target.

[0173] The sensitivity of the assay (**FIG. 43**) was calculated by measuring reaction rates
25 generated from a dilution series of PDGF-BB concentrations. The minimum detection level on the Wallac instrument was estimated at 0.8 picomoles in a 100 microliter assay volume, based upon the calculated value of three times the standard deviation of the background noise of the assay.

[0174] The assay sensitivity was also determined using PDGF-AA as a target. The aptamer
30 monomer is expected to have an affinity for PDGF-AA about ten times weaker than for PDGF-

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BB. However, since the assay involves forming a complex of two aptamer-dimers to either type of PDGF, the avidity of binding of the dimer is expected to be tighter than the affinity of the monomer, and its affinity should be substantially tighter (lower K_i) than the concentrations tested of the target PDGFs (down to about 1 nanomolar). As shown in **FIG. 44**, the reaction rates of the aptamer DPC probes to PDGF-AA at low or high concentrations (0, 1.25, 2.5, 5, 10, 20, and 40 pmole of PDGF-AA) were not substantially different than the reaction rates with PDGF-BB. This is consistent with the model of an aptamer pair binding as a dimer and exhibiting increased avidity.

[0175] *Ratios of TPP to AzC Probes.* To confirm the model of the reaction mechanism (**FIG. 8**, the optimal ratio of TPP to AzC probes would be expected to be 1:1), **FIG. 45** was an experiment in which the total amount of the two probes was kept constant, at 800 nMoles probes/reaction, while the ratio of the two probes was varied. The ratio producing the highest reaction rate was approximately 1:1, consistent with the expected mechanism.

[0176] Thus, in this model system fluorescence was not generated unless the aptamers bound and the complementary sequences in the two probes annealed to each other.

Example 19. Zip-Coded Architecture for Nucleic Acid-templated Chemistry Based-Biodetection with Aptamer Binders

[0177] **FIG. 14 [15x]** illustrates in more detail an exemplary zip-code architect. The TPP pair contained, first, a PDGF-aptamer on the 5'-end, a C18 polyethylene-glycol based spacer, and an 18-mer zip code sequence. The TPP reporter sequence contained a complementary anti-zip code sequence on its 3' terminus, a C18 PEG spacer, and a ten base pair reporter sequence terminating in a 5' TPP group. The pair of oligonucleotides comprising the AzC detection probe contained a 3'-aptamer linked through a C18 PEG spacer to a separate zip code, and a detection oligonucleotide linked to a 5' anti-zip code, a C18 PEG spacer, and a reporter oligonucleotide (complementary to the TPP oligonucleotide) terminating in a 3' AzC group.

[0178] The reaction, in 35% formamide at 22°C, was dependent upon the presence of both of the reporter oligonucleotides, both of the aptamer oligonucleotides, and the target, PDGF-BB (**FIG. 46**). At 22°C in the absence of formamide, the reaction proceeded independently of the presence of PDGF. This is consistent with the behavior of the above-described "one-piece" architect, and reflects that the mechanism of fluorescence generation in 35% formamide is dependent the increased thermal stability of the reporter sequence duplex in formamide upon

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addition of PDGF. In the absence of formamide at 22°C, the reporter oligonucleotide duplex is stable both in the presence and absence of PDGF.

[0179] Confirmation of the correctness of the model was obtained with experiments varying the ratio of the TPP and AzC aptamer oligos (**FIG. 47**). These experiments indicated that the optimal ratio of the aptamer oligos was the expected 1:1 ratio (i.e. 50% TPP oligo with a total concentration of PDGF and aptamer oligos of 0.4 µM). The optimal ratio of total reporter oligonucleotides to total aptamer oligos was also 1:1. No PDGF-dependent reaction occurred in the complete absence of either one of the reporter or aptamer oligonucleotides. At higher than stoichiometric concentrations of reporter oligonucleotides, the PDGF-independent signal increased (background) but the PDGF-dependent signal remained about constant. Both of these observations are consistent with the model that the complex is assembled in the ratio of 1:1:1 for each of the aptamer oligos, each of the reporter oligos, and PDGF.

[0180] These experiments indicate that the complex can self-assemble in solution, such that each zip code and its anti-zip code anneal to each other with minimal interference with the aptamer sequence or the reporter sequences.

[0181] Experiments were also conducted to determine if the order of addition, and thus assembly of the aptamer and reporter probes, was of any importance. Slightly slower reaction rates were obtained if the aptamer oligonucleotides were first incubated with PDGF before adding the reporter oligonucleotides, compared with adding all probes together as a mixture. Somewhat greater reaction rates were obtained if each pair of aptamer oligonucleotides and reporter oligonucleotides was first incubated and allowed to assemble with each other before the two sets were mixed together and incubated with PDGF. The reason for this may be that there is some steric hindrance to zip code-anti zip code annealing to aptamer probe if the aptamer probe is already bound to target.

[0182] As a control, a set of one-piece TPP and AzC probes was compared which contained only the zip code sequences and no zip code-anti zip code sequences (**FIG. 48**). The reaction rates of this one-piece system were similar to that of the two-piece system, except that the rate enhancement due to the addition of PDGF was typically slightly better than that of the two-piece system.

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[0183] The sequence of the aptamer-containing TPP and AzC probes was also systematically varied to determine any constraints on the design. The aptamer-containing TPP and AzC oligos were synthesized, both having the same sequences as described in FIG. 14 but with the following changes: (1) omission of the C18-PEG spacer. (Oligos 119 & 122); (2) replacement of the C18-PEG spacer with the sequence C₁₀. (oligos 120 & 123); (3) replacement of the C18-PEG spacer with the sequence C₂₀. (oligos 121&124); (4) Omission of the C18-PEG spacer and omitting 3 3'-bases in the zip code region (reduction to 15 bases in length). (oligos 127 & 129); and (5) omission of the C18-PEG spacer and omitting 6 3'-bases in the zip code region (reduction to 12 bases in length). (oligos 128 & 130).

[0184] Oligonucleotides used in this example included:

Oligo#	Sequence (5'-3')	Modification
--------	------------------	--------------

(SEQ. ID NO. 43)

106 GGACTCGAGCACCAATAC-X-TATAAATTCG-AZC X= C18 PEG; AZC = 3'- AzC.

(SEQ. ID NO. 44)

109 CGAATTATATA-X-CTGACCATCGATGGCAGC X=C18 PEG, 5'-TPP

(SEQ. ID NO. 45)

112 CAGGCTACGGCACGTAGAGCATCACCATGATCCTG-X-GCTGCCATCGATGGTCAG X= C18 PEG

(SEQ. ID NO. 46)

113 GTATTGGTGCTCGAGTCC-X-CAGGCTACGGCACGTAGAGCATCACCATGATCCTG X= C18 PEG

(SEQ. ID NO. 47)

119 GTATTGGTGCTCGAGTCCCAGGCTACGGCACGTAGAGCATCACCATGATCCTG

(SEQ. ID NO. 48)

120 GTATTGGTGCTCGAGTCCCCCCCCCCCCCAGGCTACGGCACGTAGAGCATCACCATGATCCTG

(SEQ. ID NO. 49)

121

GTATTGGTGCTCGAGTCCCCCCCCCCCCCCCCCCCCCCCCCAGGCTACGGCACGTAGAGCATCACCATGATCCTG

(SEQ. ID NO. 50)

122 CAGGCTACGGCACGTAGAGCATCACCATGATCCTGGCTGCCATCGATGGTCAG

(SEQ. ID NO. 51)

123 CAGGCTACGGCACGTAGAGCATCACCATGATCCTGCCCCCCCCCGCTGCCATCGATGGTCAG

(SEQ. ID NO. 52)

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124

CAGGCTACGGCACGTAGAGCATCACCATGATCCTGCCCCCCCCCCCCCCCCCGCTGCCATCGATG
GTCAG

(SEQ. ID NO. 53)

5 127 CAGGCTACGGCACGTAGAGCATCACCATGATCCTGGCTGCCATCGATGGT

(SEQ. ID NO. 54)

128 CAGGCTACGGCACGTAGAGCATCACCATGATCCTGGCTGCCATCGAT

(SEQ. ID NO. 55)

129 TTGGTGCTCGAGTCCCAGGCTACGGCACGTAGAGCATCACCATGATCCTG

10 (SEQ. ID NO. 56)

130 GTGCTCGAGTCCCAGGCTACGGCACGTAGAGCATCACCATGATCCTG

[0185] None of these changes resulted in a significant difference in the performance of the system. Experiments 4) and 5) also resulted in a 3 and 6-base single stranded (not annealed to zip code) structure immediately upstream of the C18 spacer in the reporter oligonucleotides.

15 **[0186]** The results of these experiments indicate that the aptamer-based PDGF detection system can be assembled separating the binding and DPC functions into two separate oligonucleotides. Through the selection of appropriate zip code sequences, the detection format described in **FIG. 13** self-assembled into pairs of annealed oligonucleotides which will function similarly to oligonucleotides synthesized in a single piece. The reporter and aptamer
20 oligonucleotides may be separately assembled prior to introduction of target, or all species may be added together in almost any order. This process may be extended to the solution-phase assembly of more than one pair of annealed detection oligos, for example, to detect multiple targets. Detection of multiple targets may require using different reporter oligonucleotides which generate separately discernable signals (for example, different wavelengths of emitted
25 light).

[0187] These results indicate that a zip-coded reporting approach can be effectively designed, for example, using aptamer-containing oligonucleotides.

[0188] While the results with the aptamer system indicate that a stable complex between binding and reporter sequences can be formed simply by annealing the zip code and anti-zip
30 code regions, it should be noted that there are technologies to covalently and irreversibly link the two oligonucleotides together, with a high likelihood of retaining activity of the reporter reactive groups. For example, the oligonucleotides may be incubated in pairs (a binder oligonucleotide and a reactive oligonucleotide for nucleic acid-template chemistry) at a

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temperature at which the zip codes and anti-zip codes are mostly double stranded, but the rest of the sequences are single-stranded. Adding an intercalating, photoactivatable cross-linker such as Trioxalen, followed by UV irradiation, may irreversibly crosslink the two strands. Similarly, UV irradiation may introduce thymidine dimers between separate strands of annealed sequences. Alternately, a sequence may be introduced complementary to a short target (splice) DNA, abutting 3' and 5', which may then be ligated with DNA ligase. The splice oligonucleotide may alternately be composed of RNA, and removed after ligation with RNase H, which hydrolyzes RNA annealed to DNA. This can result in converting the two oligonucleotides into a single piece of single-stranded DNA. These methods can lead to cost-effective production of oligonucleotide reagents in detection kits against specific targets.

[0189] Relevant references for this example include Capaldi, *et al.*, Nucleic Acid Res. **2000**, 28[7], e21.; Castiglioni, *et al.*, Appl. and Exper. Microbio. **2004**, 7161-72; Fang, *et al.*, Chem.BioChem. **2003**, 4, 829-34.; Gerry, *et al.*, J. Mol. Biol. **1999**, 292, 251-62.

Example 20. Zip-Coded Architecture for DPC-based Biodetection – Antibody Binders

[0190] In another embodiment, the aptamer sequences are replaced with non-DNA binders such as antibodies. For PDGF and other protein targets, the aptamer sequences are replaced with chemically active groups, such as aldehydes, and reacted with non-DNA binder sequences such as antibodies or receptors to the protein targets (**FIG. 16**). The optimal design for the binder and reporter oligonucleotides may be achieved with considerations on the size and geometry of the binder and size and geometry of the binding sites of the target. A longer, or shorter spacer arms, for example, may be used to optimally span the distance between binding sites on the target and avoid steric hindrance due to the binders themselves.

[0191] Referring to **FIG. 16**, the zip-coded oligonucleotide designed to hybridize to the TPP reporter molecule was synthesized containing a 5'-amino group. The zip-coded oligonucleotide designed to hybridize to the AzC reporter molecule contained a 3'-amino group. Synthesis of the conjugates between the oligonucleotides and anti-PDGF-BB antibody were performed by SoluLink Biosciences (San Diego, CA).

[0192] The SoluLink technology for conjugation of the antibody and oligonucleotides first requires modification of the primary amino groups of the antibody with succinimidyl 2-hydrazinonicotinate acetone hydrazone) to incorporate an acetone hydrazone onto the antibody.

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The primary amines of the oligonucleotides are separately activated with succinimidyl 4-formylbenzoate. The two activated molecules are mixed in the desired ratio (typically 6:1) and reacted at a mildly acidic pH to form a stable hydrazone linkage. The details of this chemistry are available at www.SoluLink.com. Two conjugates were prepared: one containing the zip code to anneal to the AzC-containing reporter oligonucleotide, and the other containing the zip code to anneal to the TPP-containing reporter oligonucleotide.

[0193] The antibody-oligonucleotide conjugates received from SoluLink were further purified by gel chromatography on a 1.6 x 60 cm column of Superdex S-200 (Amersham Biosciences) in PBS buffer (0.01 M potassium phosphate, pH 7.4 – 0.138 M sodium chloride).

The main antibody peak, eluting at about 0.6 times the column volume, was collected and a later eluting peak of contaminating non-conjugated oligonucleotide was discarded. The antibody conjugate was concentrated by reversed dialysis with a Pierce (Rockford, IL) 30 K molecular weight cut-off Slide-A-Lyzer using Pierce Concentrating Solution. The protein content was determined using the Bio-Rad Micro BCA Reagent Kit and the oligonucleotide content determined using SYBR Gold DNA binding dye (Molecular Probes (Eugene, OR). The conjugates were both determined to contain an average of approximately 3 oligonucleotides per protein molecule.

[0194] Recombinant human PDGF-BB (220-BB) and mouse monoclonal anti-PDGF-BB (MAB220) were obtained from R&D Systems (Minneapolis MN).

[0195] Sequences used in this study included (where AzC indicates azidocoumarin and TPP indicates triphenylphosphine):

<u>Name</u>	<u>Sequence (5'-3')</u>
-------------	-------------------------

TPP reporter	TPP-(amino modifier C6)-CGAATTTATA-C18PEG-TCAGCATCGTACCTCAGC (SEQ ID NO.: 9) (SEQ ID NO.: 58)
--------------	--

AzC reporter	GGACTCGAGCACCAATAC-C18 PEG-TATAAATTCG-(amino modifier C7)-AzC (SEQ ID NO.: 14) (SEQ ID NO.: 10)
--------------	--

AzC zip code	TTGGTGCTCGAGTCCCCCCCCCCCCCCCCCCCCC-(amino modifier C7) (SEQ ID NO.: 59)
--------------	--

TPP zip code	(amino modifier C6)-CCCCCCCCCCCCCCCCCCCCCGCTGAGGTACGATGCTGA (SEQ ID NO.: 60)
--------------	---

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[0196] In addition, the 5' amino modifier C6 was obtained from Glen Research (from Glen Research phosphoramidite 110-1906). The 3'-amino modifier C7 was obtained from Glen Research (from Glen Research CPG 20-2957). The C18 PEG was obtained from Glen Research (from Glen Research phosphoramidite 10-1918).

5 Assembly of antibody-oligo conjugates with reporter oligonucleotides.

[0197] The two antibody-oligo conjugates with their reporter were first assembled separately in a volume of 10 μ l. Each assembly contained 0.5 μ M (5 picomoles) of antibody-oligonucleotide conjugate and 0.15 μ M of (15 pmoles) of complementary reporter oligonucleotide in 0.05 M Tris/HCl pH 8 – 0.01 M magnesium chloride. Each was incubated
10 for at least 15 minutes at 4° C before use in the detection reaction mixture.

Detection Reaction of anti-PDGF-BB DPC Conjugates/Reporters with PDGF-BB

[0198] To conduct detection reaction, each reaction may contain in a volume of 50 μ l: 10 μ l of each conjugate assembly, prepared as described above, and variable amounts of PDGF-BB, in a buffer of 0.05 M Tris/HCl pH 8 – 0.01 M magnesium chloride-40% volume/volume
15 formamide. The conjugates are present in this reaction mixture at 0.2 μ M. Samples are incubated in the wells of a black 96-well microplate in a Wallac Victor Luminometer at 25° C. Fluorescence can be followed vs. time with excitation at 355 nm and emission at 460 nm.

[0199] Reactions typically may be carried out at 25° C, monitoring fluorescence generation at the wavelength optimums of the reaction product, 7-amino coumarin.

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INCORPORATION BY REFERENCE

[0200] The entire disclosure of each of the publications and patent documents referred to herein is incorporated by reference in its entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

EQUIVALENTS

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[0201] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing

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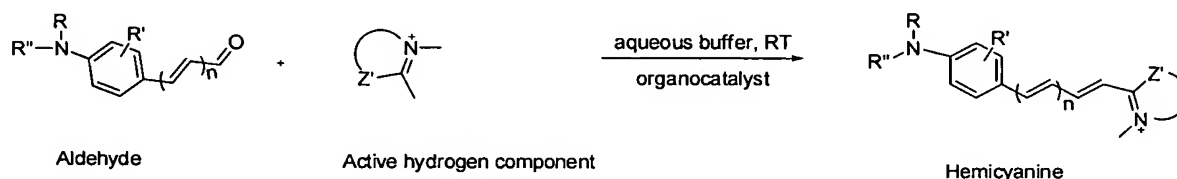
description, and all changes that come within the meaning and range of equivalency of the claims are intended to be embraced therein.

[0202] What is claimed is:

CLAIMS

1. A method for making a polymethine dye comprising conducting an aldol condensation between an aldehyde and an active hydrogen component in an aqueous condition in the presence of an organocatalyst.

2. The method of Claim 1 wherein the condensation reaction is:



wherein

$Z' = O, S, Se, P, NH_2, NR, C(CH_3)_2$ where R is alkyl group

$n = 0, 1, 2 \dots$

$R = H, \text{alkyl}$

$R'' = H, \text{alkyl, alkyl carboxylic acid}$

$R' = Ph \text{ or } N\text{-heterocycle, } H, \text{alkyl, } SO_3H, OH, CN, Cl, Br, NO_2, NH_2, N(R)_2, OR \text{ where R is alkyl group}$

and wherein the organocatalyst is a secondary amine, a primary amine, or a bifunctional amine-acid catalyst.

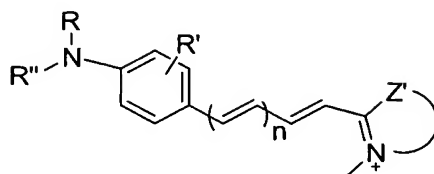
3. The method of Claim 2 wherein the secondary amine is a pyrrolidine, a piperidine, a normicotine, or an analog thereof.

4. The method of Claim 2 wherein the primary amine is a valine or a peptide having fewer than 3 amino acid units.

5. The method of Claim 2 wherein the bifunctional amine-acid catalyst is pyrrolidine/AcOH.

6. The method of Claim 2 wherein the organocatalyst is pyrrolidinylmethyl-pyrrolidine, aminomethyl pyrrolidine, dimethylethane-1,2-diamine, propane-1,2-diamine, 1-(2-aminoethyl)-piperidine, or diethylethylene-1,2-diamine.

7. A hemicyanine dye having the chemical structure of (I) prepared by the method of Claim 2.



Wherein

(I)

Z' = O, S, Se, P, NH₂, NR, C(CH₃)₂ where R is alkyl group

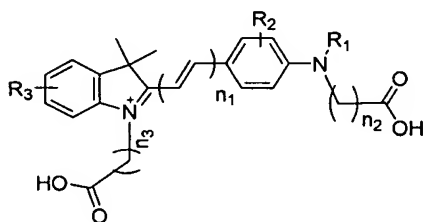
n = 0, 1, 2 ...

R = H, alkyl

R'' = H, alkyl, alkyl carboxylic acid

R' = Ph or N-heterocycle, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂, NH₂, N(R)₂, OR where R is alkyl group.

8. A hemicyanine dye having the chemical structure of II or III:



n1 = 1, 2, 3, 4, 5

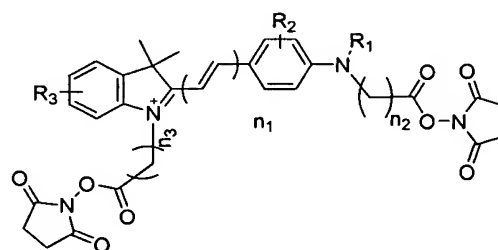
n2, n3 = 0 to 16

R1 = alkyl

R2 = Ph, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂, NH₂, N(R₁)₂, OR₁ while R₁ is alkyl group

R3 = Ph, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂, NH₂, N(R₁)₂, OR₁ while R₁ is alkyl group

(II)



n1 = 1, 2, 3, 4, 5

n2, n3 = 0 to 16

R1 = alkyl

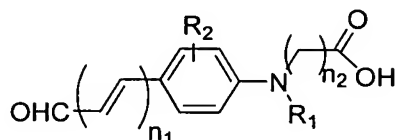
R2 = Ph, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂, NH₂, N(R₁)₂, OR₁ while R₁ is alkyl group

R3 = Ph, H, alkyl, SO₃H, OH, CN, Cl, Br, NO₂, NH₂, N(R₁)₂, OR₁ while R₁ is alkyl group

(III)

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- 1 9. An aldehyde having the chemical structure of IV or V:



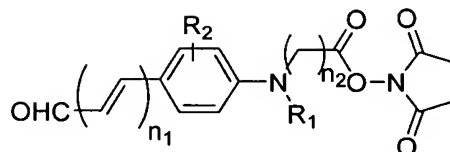
$n_1 = 1, 2, 3, 4, 5$

$n_2 = 0 \text{ to } 16$

$R_1 = \text{alkyl}$

$R_2 = \text{Ph, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1 \text{ while } R_1 \text{ is alkyl group}$

(IV)



$n_1 = 1, 2, 3, 4, 5$

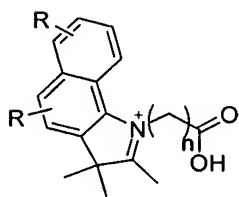
$n_2 = 0 \text{ to } 16$

$R_1 = \text{alkyl}$

$R_2 = \text{Ph, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1 \text{ while } R_1 \text{ is alkyl group}$

(V)

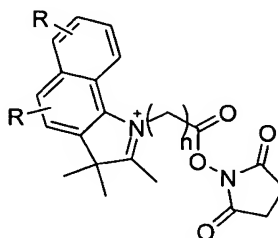
- 1 10. A quaternary salt having the chemical structure of VI or VII:



$n = 0 \text{ to } 16$

$R = \text{H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1 \text{ while } R_1 \text{ is alkyl group}$

(VI)

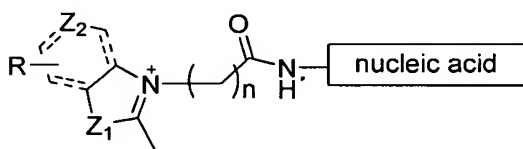


$n = 0 \text{ to } 16$

$R = \text{H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1 \text{ while } R_1 \text{ is alkyl group}$

(VII)

- 1 11. A quaternary salt-nucleic acid conjugate having the chemical structure of:



$n = 0 \text{ to } 16$

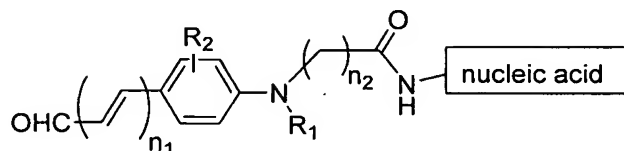
$Z_1 = \text{O, S, Se, P, NH}_2, \text{NR}_1, \text{C(CH}_3)_2 \text{ where } R_1 \text{ is alkyl group}$

$R = \text{any substituted benzyl or higher fused benzyl rings, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1 \text{ while } R_1 \text{ is alkyl group}$

$Z_2 = \text{benzene or any N-heterocycles}$

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12. An aldehyde-nucleic acid conjugate having the chemical structure of:



$n_1 = 1, 2, 3, 4, 5$

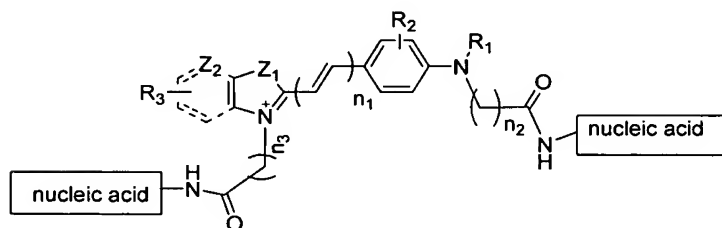
$n_2 = 0 \text{ to } 16$

$R_1 = \text{H, alkyl}$

$R_2 = \text{Ph or N-heterocycle, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1$

while R_1 is alkyl group

13. A hemicyanine dye-nucleic acid conjugate having the chemical structure of:



$n_1 = 1, 2, 3, 4, 5$

$n_2 \text{ and } n_3 = 0 \text{ to } 16$

$Z_1 = \text{O, S, Se, P, NH}_2, \text{NR}_1, \text{C(CH}_3)_2$

where R_1 is alkyl group

$R_3 = \text{any substituted benzyl or higher}$

fused benzyl rings, H, alkyl, $\text{SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1$ while

R_1 is alkyl group

$Z_2 = \text{benzene or any N-heterocycles}$

$R_1 = \text{H, alkyl}$

$R_2 = \text{Ph or N-heterocycle, H, alkyl, SO}_3\text{H, OH, CN, Cl, Br, NO}_2, \text{NH}_2, \text{N(R}_1)_2, \text{OR}_1$

while R_1 is alkyl group

14. A method for making a hemicyanine-nucleic acid conjugate comprising conducting a nucleic acid-templated reaction between an aldehyde of Claim 12 and quaternary salt of Claim 11 to make a hemicyanine of Claim 13.

15. The method of Claim 14 wherein the nucleic acid-templated reaction is in an end of helix architect.

16. The method of Claim 14 wherein wherein the nucleic acid-templated reaction is in a middle of helix architect.

17. A method for selecting a dye having a desired fluorescent property, the method comprising:

(a) preparing a library of oligonucleotide-encoded dyes through nucleic acid-templated synthesis;

(b) hybridizing the oligonucleotide-encoded dyes with spatially arrayed complementary oligonucleotide probes immobilized on a solid support;

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7 (c) measuring the absorption and fluorescence properties of the oligonucleotide-
8 encoded dye directly on the solid support.

9 (d) identifying the oligonucleotides that encode the dyes having the desired
10 fluorescence properties based on the position of the immobilized complementary
11 oligonucleotide probes, and

12 (e) identifying and characterizing the chemical structure of the dyes having the
13 desired fluorescence property.

1 18. A method for detecting multiple target nucleotide sequences, the method comprising:

2 (a) providing a number of probe pairs, the number equal to the number of target
3 nucleotide sequences, wherein each probe pair comprises (1) a first probe comprising (i) a first
4 oligonucleotide sequence and (ii) a first reactive group linked to the first oligonucleotide
5 sequence, and (2) a corresponding second probe comprising (i) a second oligonucleotide
6 sequence and (ii) a second reactive group linked to the second oligonucleotide sequence,
7 wherein the first oligonucleotide sequence and the second oligonucleotide sequence are
8 complementary to two separate regions of a corresponding target nucleotide sequence;

9 (b) combining the probe pairs with a sample to be tested for the presence of the
10 target nucleotide sequences under conditions where the first probes and the second probes
11 hybridize to their respective complementary regions of the target nucleotide sequences if
12 present in the sample thereby bringing into reactive proximity the first reactive groups and the
13 corresponding second reactive groups; and

14 (c) detecting one or more reactions between the first reactive groups and the
15 corresponding second reactive groups thereby determining the presence of the target nucleotide
16 sequences.

1 19. The method of Claim 18 wherein the number of target nucleotide sequences is 2 to 20.

1 20. The method of Claim 18 wherein the number of target nucleotide sequences is 2 to 6.

1 21. The method of Claim 18 wherein the target nucleotide sequences are in solution phase.

1 22. The method of Claim 18 wherein the target nucleotide sequences are attached to a solid
2 support.

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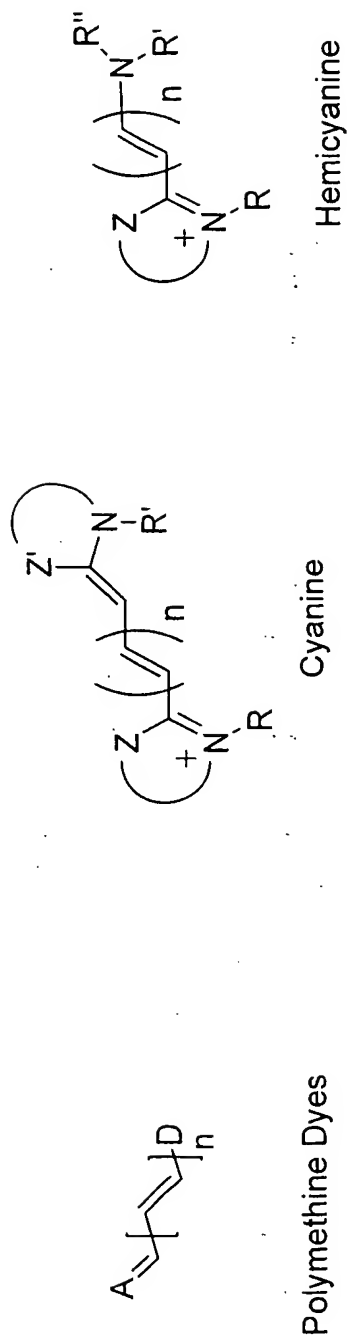
1 23. The method of Claim 18 wherein the one or more reactions between the first reactive
2 groups and the corresponding second reactive groups generate fluorescent compounds that may
3 be detected.

1 24. The method of Claim 18 wherein the one or more reactions between the first reactive
2 groups and the corresponding second reactive groups generate chemiluminescent compounds
3 that may be detected.

1 25. The method of Claim 18 wherein the one or more reactions between the first reactive
2 groups and the corresponding second reactive groups comprise an aldol condensation reaction.

1 26. The method of Claim 18 wherein the one or more reactions between the first reactive
2 groups and the corresponding second reactive groups comprise a Wittig reaction.

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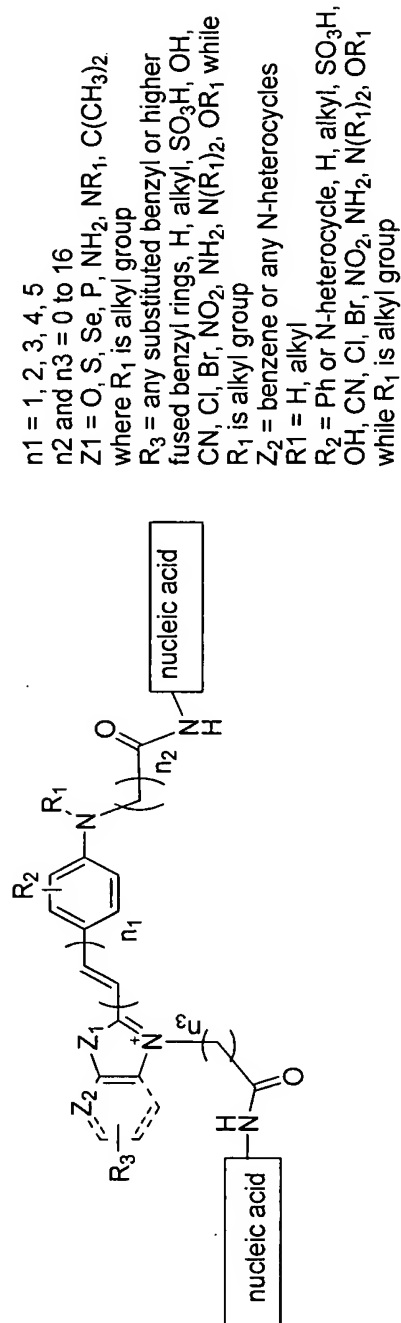
A = acceptor D = donor Z, Z' = O, S, N, C ... n = 0, 1, 2 ... R, R', R'' = alkyl chain

Typical A and D for polymethine dyes: thiazoles, pyrroles, pyrrolines, indoles, 1,3,3-trimethylindolines, tetrazoles, pyrimidine, pyridines, quinolines and higher fused N-heterocycles or any substituted benzyl rings.

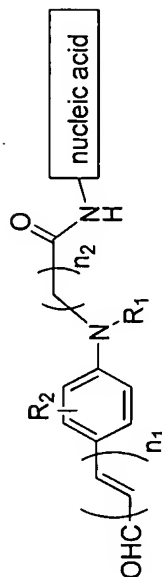
FIG. 1: General structure of polymethine dye, cyanine and hemicyanine

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Hemicyanine dye:



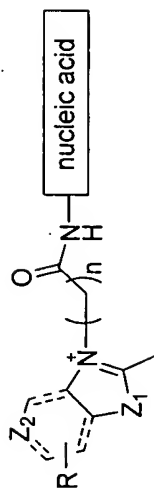
Aldehyde precursor:


 $n_1 = 1, 2, 3, 4, 5$
 $n_2 = 0$ to 16

 $R_1 = H$, alkyl

 $R_2 = Ph$ or N-heterocycle, H, alkyl, SO_3H , OH, CN, Cl, Br, NO_2 , NH_2 , $N(R_1)_2$, OR_1 while R_1 is alkyl group

Quaternary salt precursor:


 $n = 0$ to 16

 $Z_1 = O, S, Se, P, NH_2, NR_1, C(CH_3)_2$ where R_1 is alkyl group

 $R =$ any substituted benzyl or higher fused benzyl rings, H, alkyl, SO_3H , OH, CN, Cl, Br, NO_2 , NH_2 , $N(R_1)_2$, OR_1 while R_1 is alkyl group

 $Z_2 =$ benzene or any N-heterocycles

FIG. 2: General chemical structures of hemicyanine dyes useful for multiplex and their aldehyde and quaternary salt precursors.

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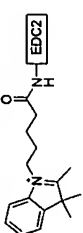
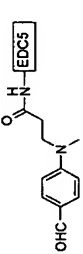
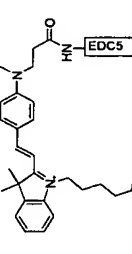

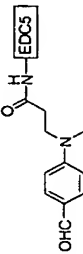
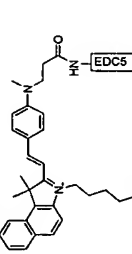
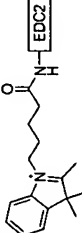
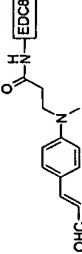
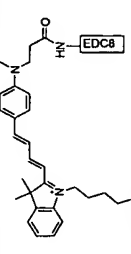
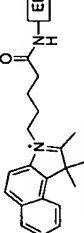
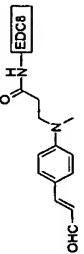
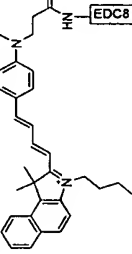
Quaternary salt bearing active hydrogen component	Aldehyde	Hemicyanine Dye	λ_{abs} (nm)	λ_{em} (nm)
 Indolinium (I)	 Aldehyde (A0)	 I H1_A0	560	585
 Benzoindolinium (BI)	 Aldehyde (A0)	 BI H1_A0	560	602
 Indolinium (I)	 Aldehyde 1 (A1)	 I H2_A1	600	670
 Benzoindolinium (BI)	 Aldehyde 1 (A1)	 BI H2_A1	620	685

FIG. 3: Chemical structures of a four-plex hemicyanine_DNA dyes and their spectroscopic properties.

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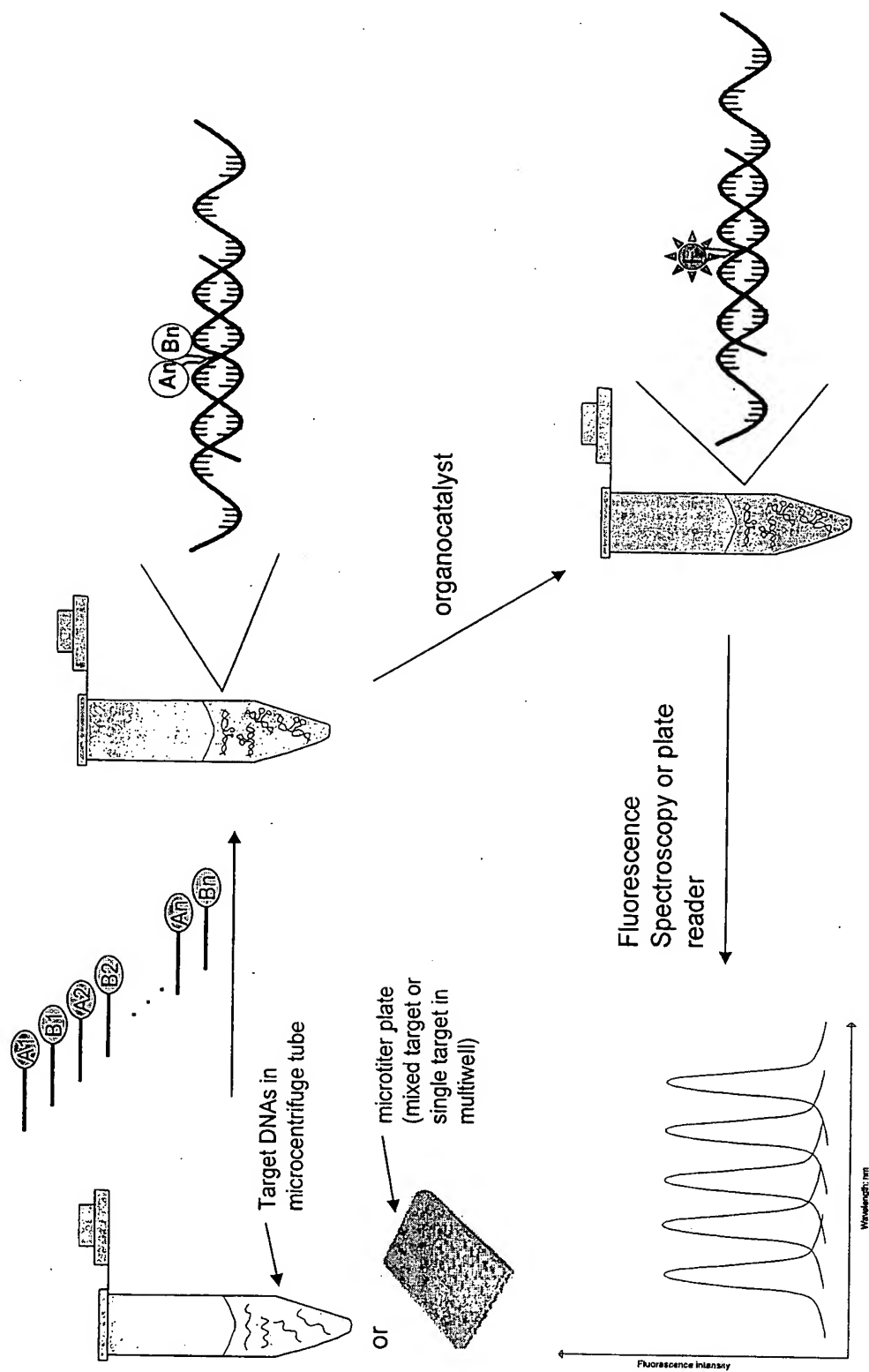


FIG. 4: Solution phase based DPC fluorescence assay for multiple analytes.

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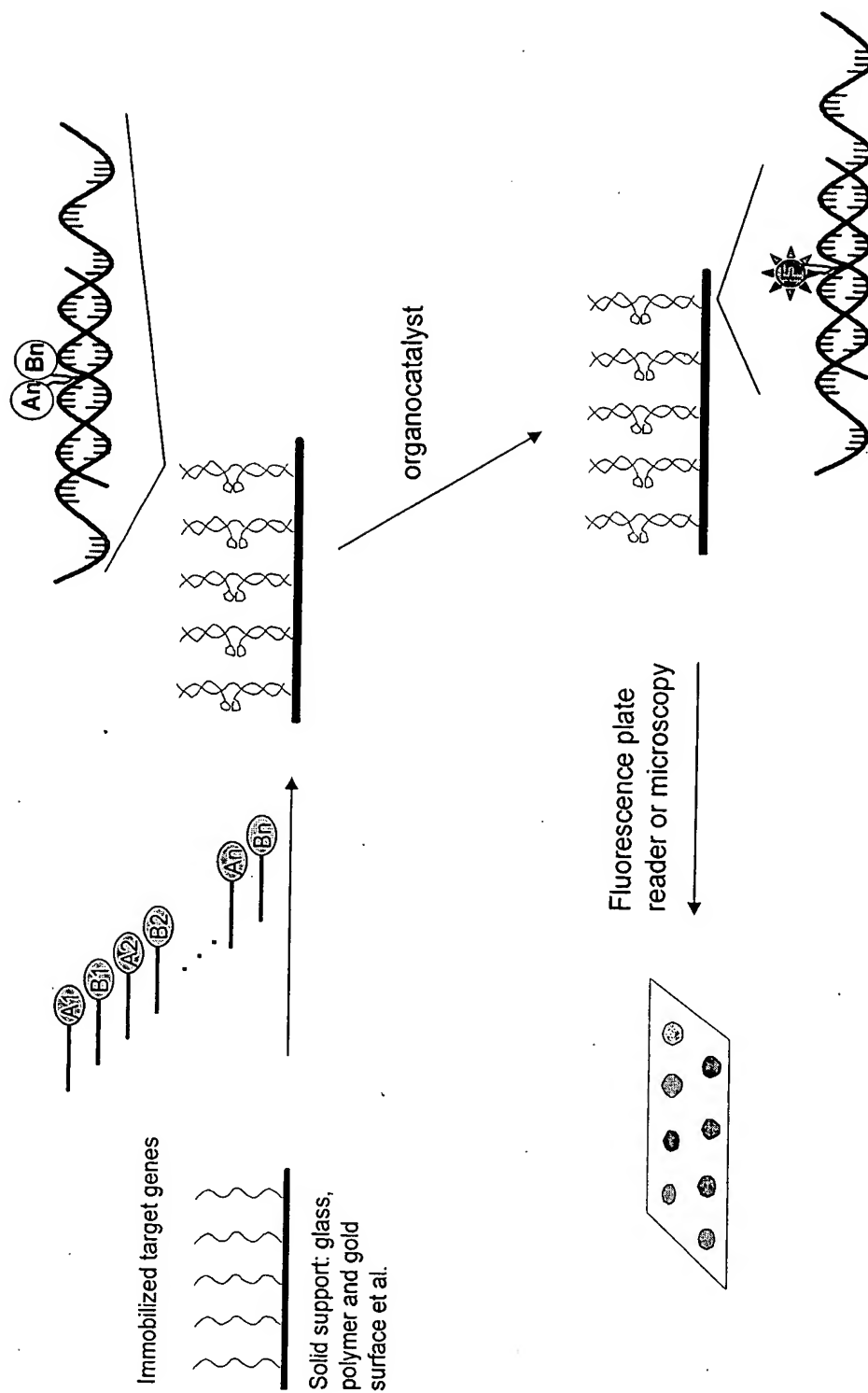


FIG. 5: Solid phase based DPC fluorescence assay for multiple analytes

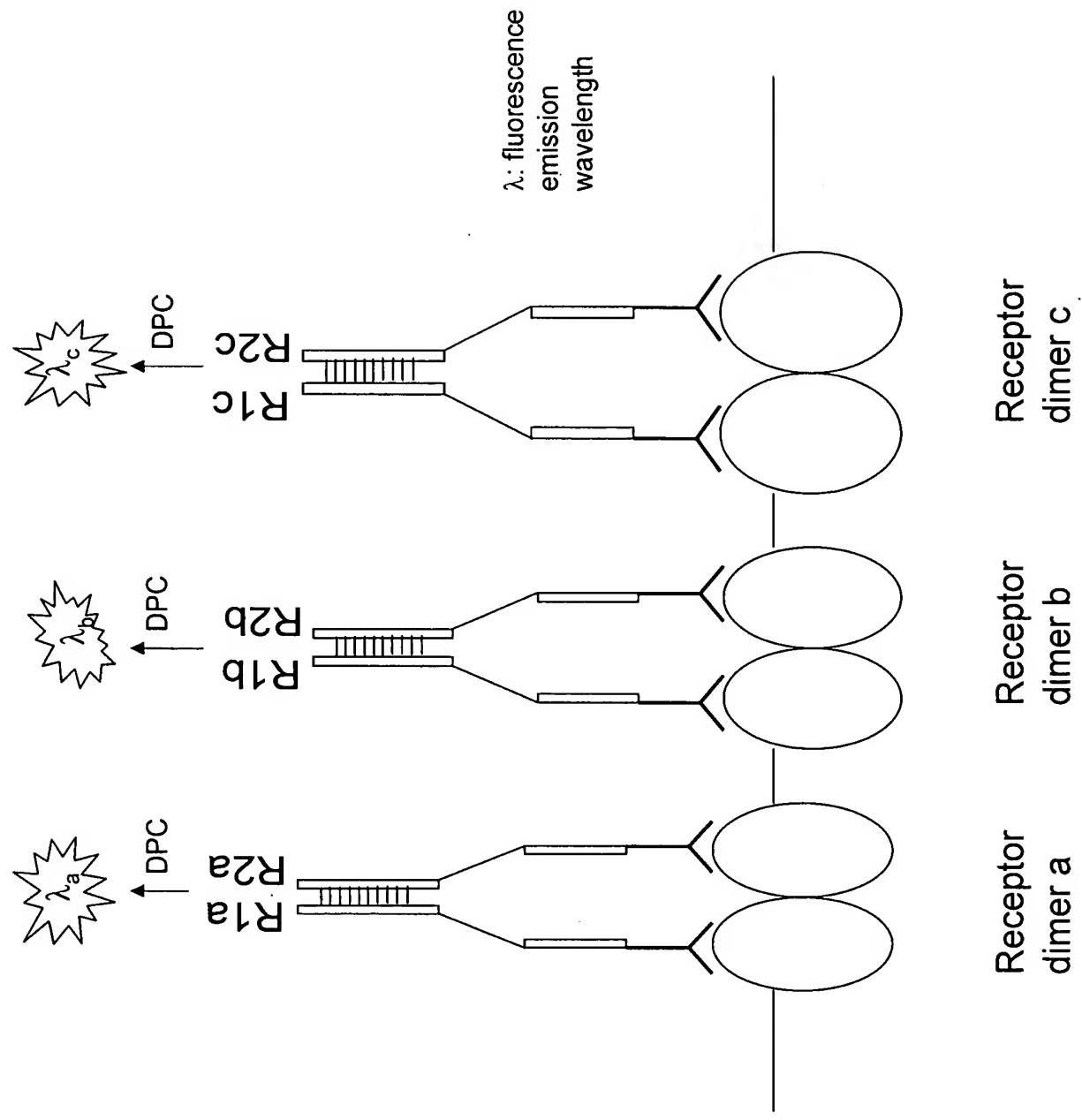


FIG. 6: Multiplexed IHC test for multiple family receptors dimer (non-zip-coded).

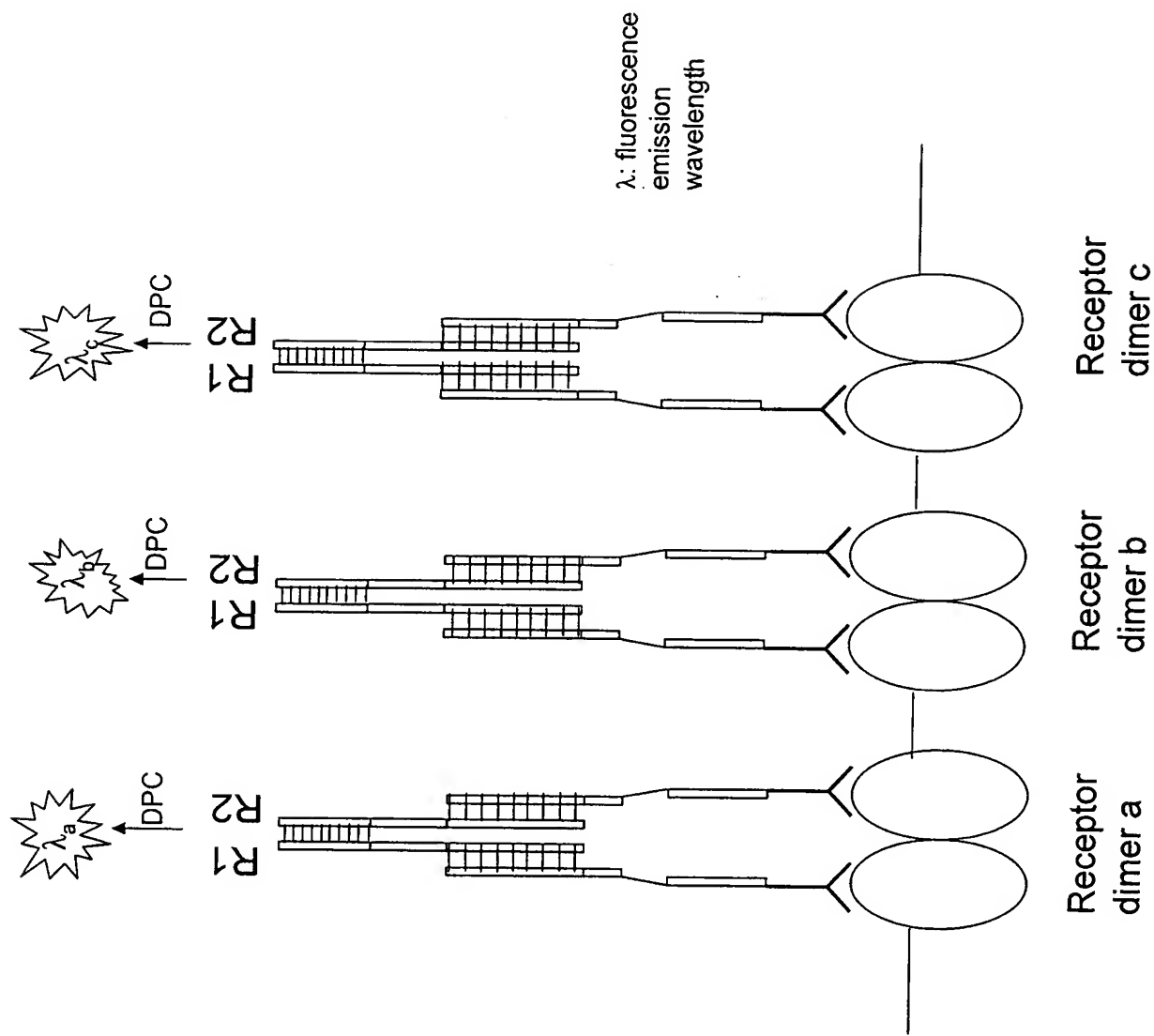
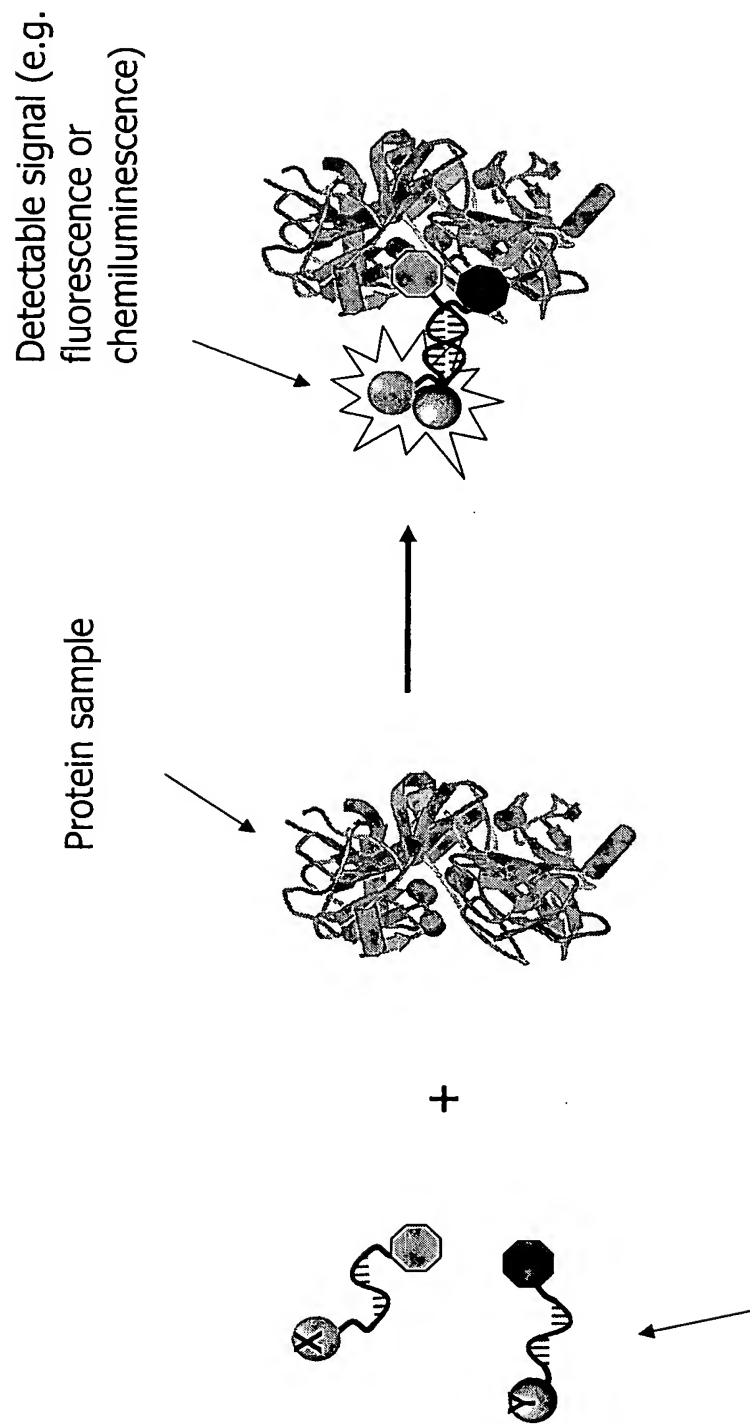


FIG. 7: Multiplexed IHC test for multiple family receptors dimer (zip coded).

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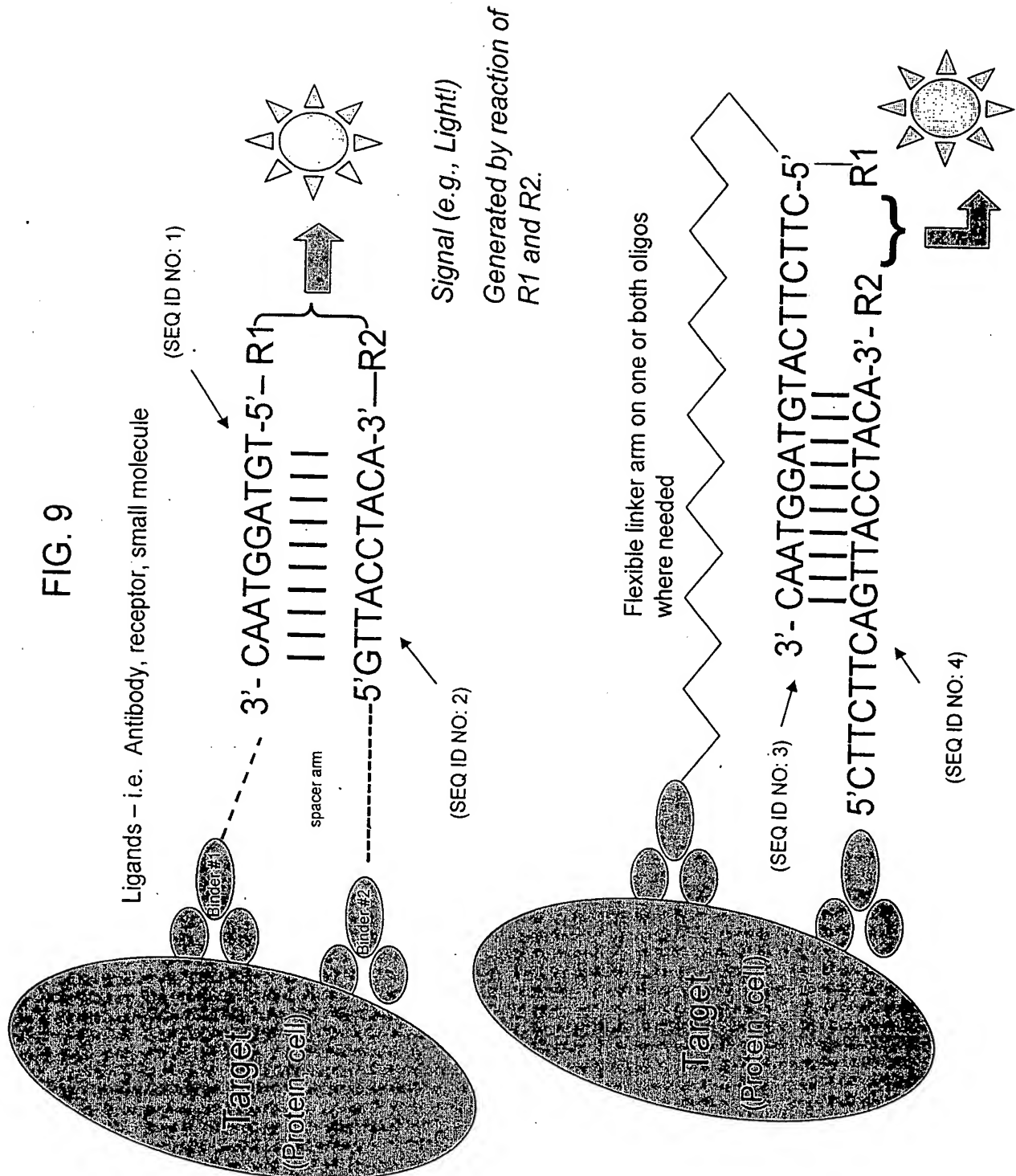
FIG. 8

Protein Detection

- Probe pair with antibody, aptamer or small molecule-based protein binding moieties and reactive groups
- Complementary oligonucleotides template detectable reaction between reactive groups

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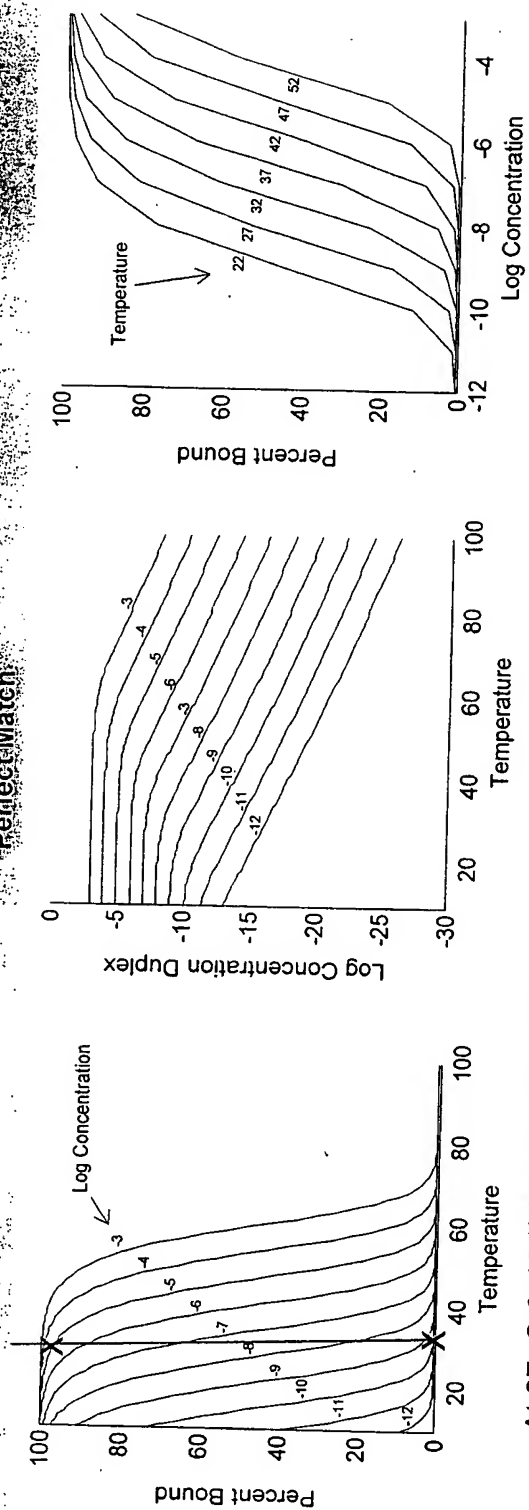
FIG. 9



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FIG. 10

Perfect Match



At 37°C & 10^{-10} M, 1% hybridized.

At 10^{-5} M, 99% hybridized

Single Mismatch

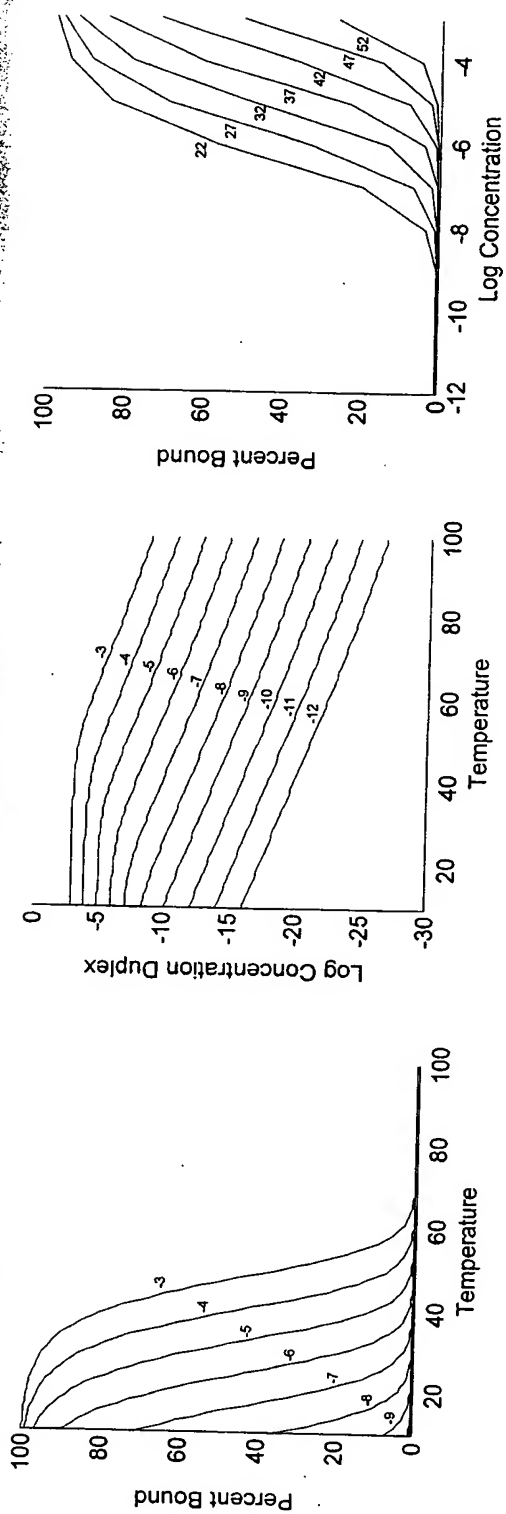


FIG. 11

Oligos Used to Construct Melting Curves

Perfect Match:

5' (+) CAATGGATGTTCTTC (SEQ ID NO: 5)

|||||

(-) CTTCTCAGTTACCTACA 5' (SEQ ID NO: 6)

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(SEQ ID NO: 7)

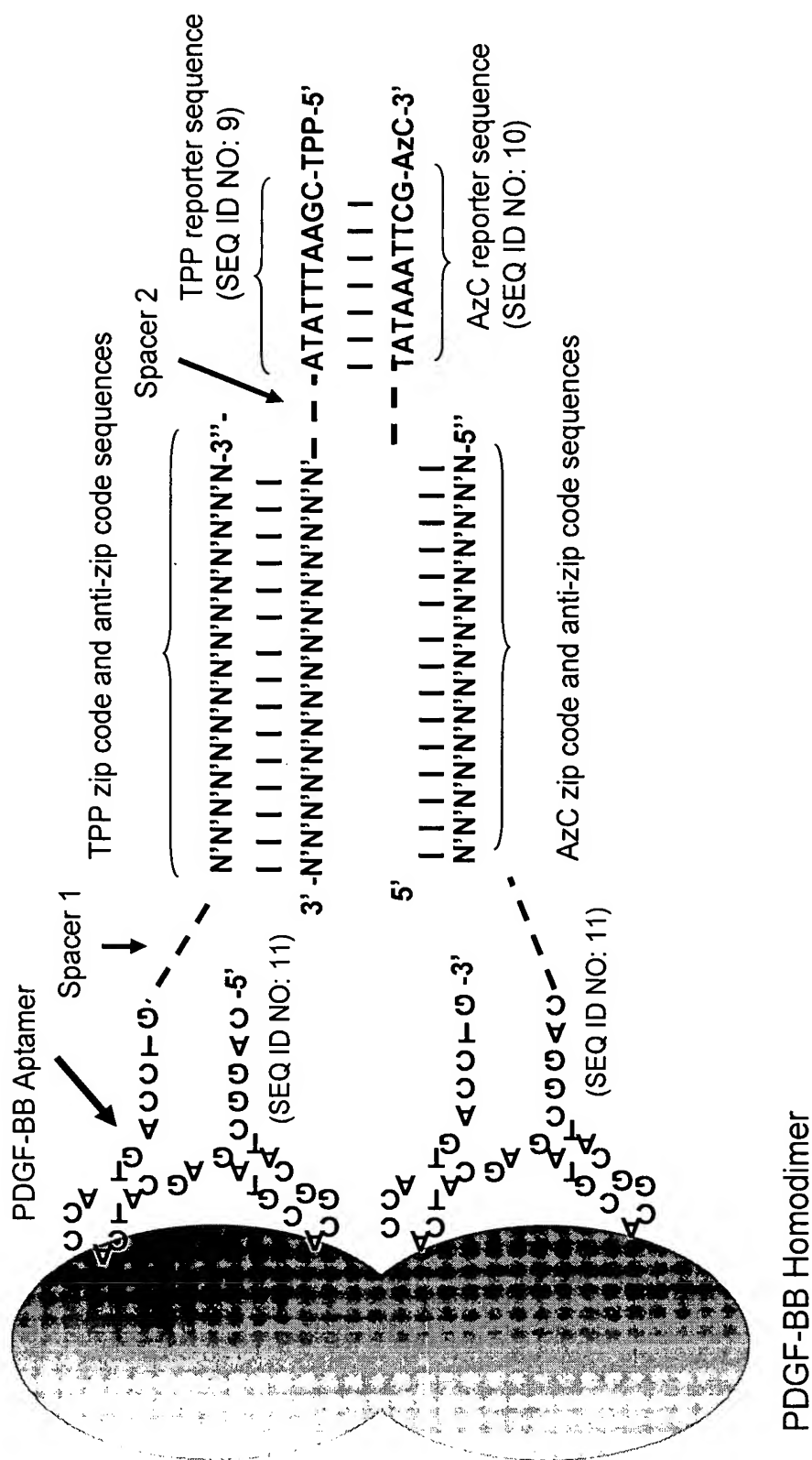
Single Mismatch:

5' (+) CAATGGATGTTCTTC (SEQ ID NO: 8)

|||||

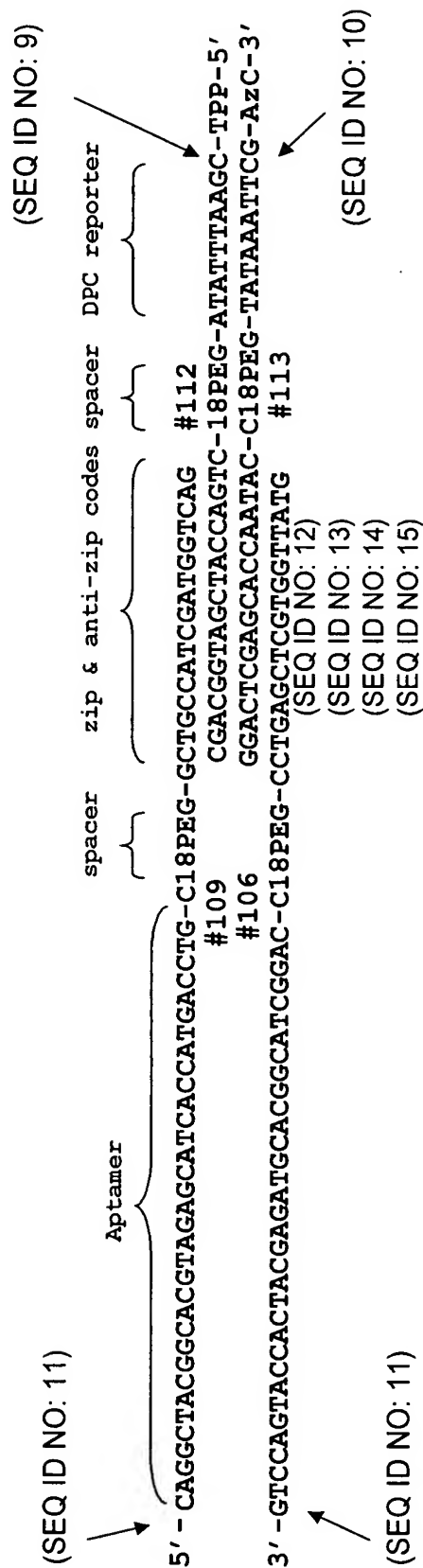
(-) CTTCTCAGTTACCTACA 5' (SEQ ID NO: 8)

FIG. 13



- Illustrative exemplary of a Zip-Coded Splinted Architecture
- NNN... represents zip code sequence, and N'N'N'... represent complement of the zip code sequence (anti-zip codes)

FIG. 14

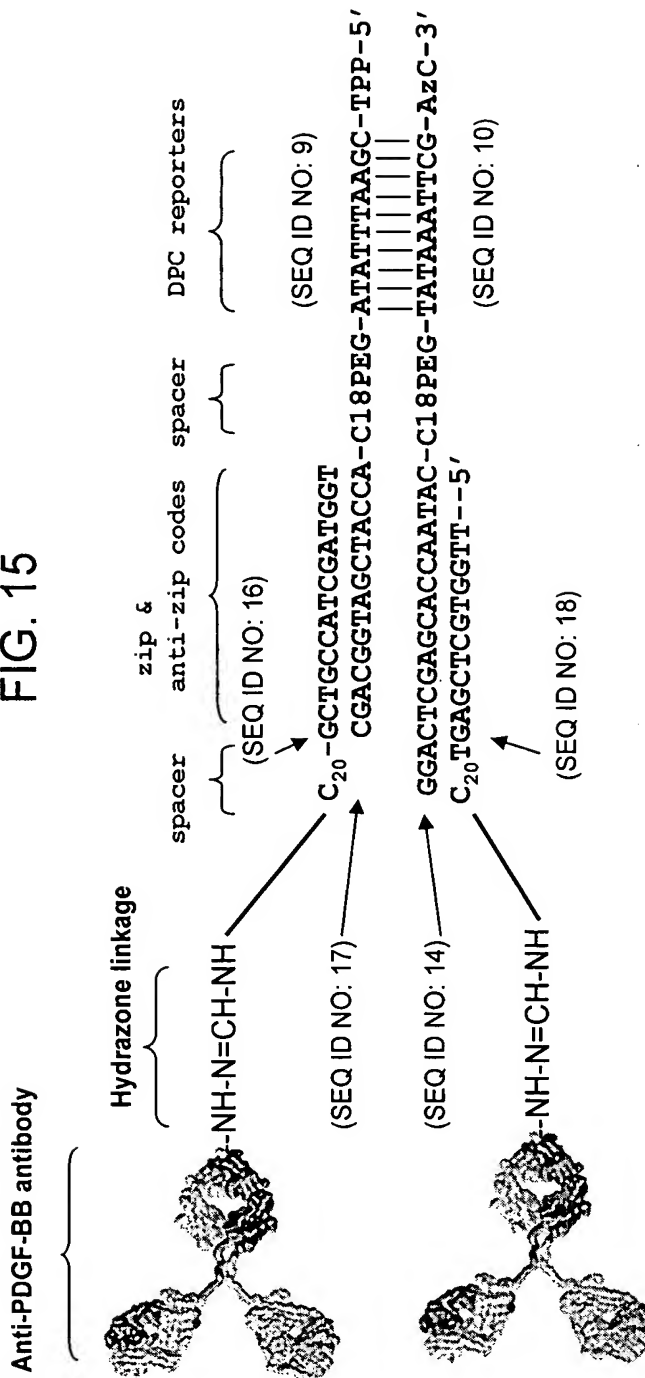


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Example of splinted, zip-coded detection probes using aptamer binders

- The upper and lower two oligonucleotides include aptamer sequence linked to separate zip code sequences with a C18 PEG spacer.
- The inner two oligonucleotides (reporter oligonucleotides) include anti-zip codes (sequences each complementary to a zip code in one of the upper and lower oligonucleotides) linked through a C18 PEG spacer to a reporter oligonucleotide.
- One reporter oligonucleotide contains a 5'-terminal TPP residue, the other a 3' terminal AzC residue. Each zip code is complementary only to its anti-zip code.
- The reporter sequences are complementary only to each other.

FIG. 15



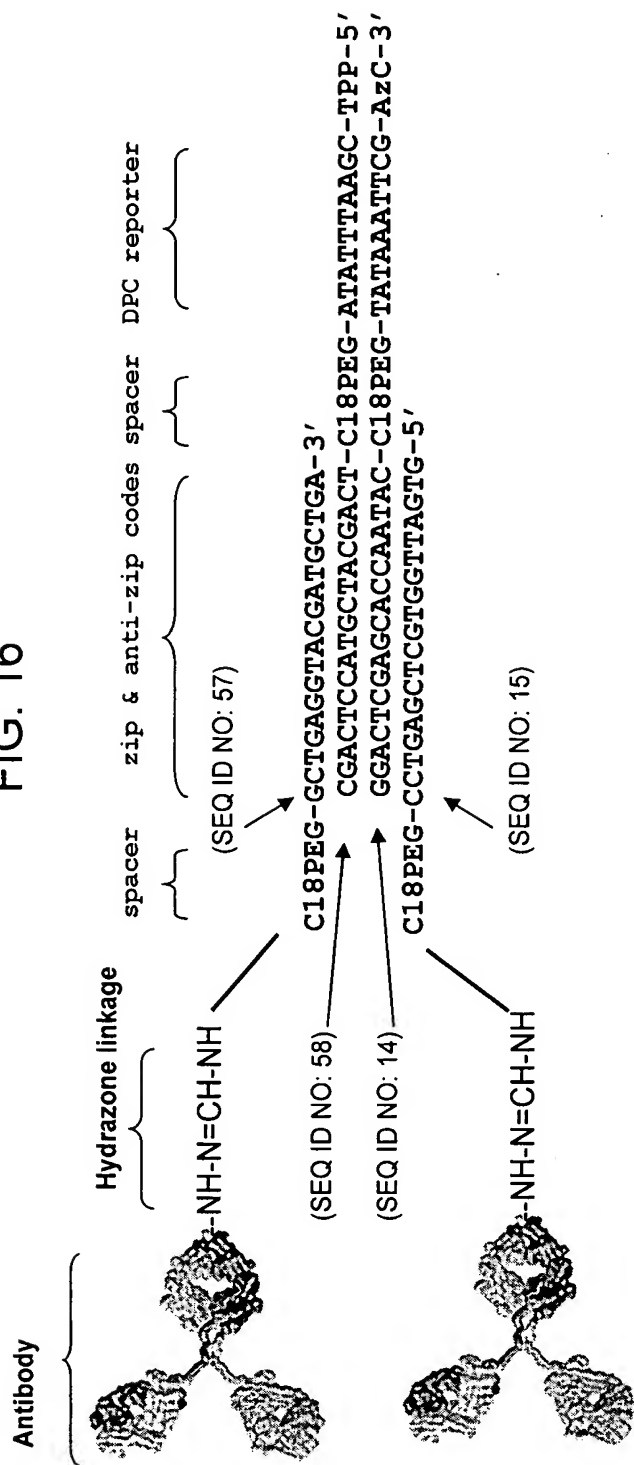
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Example of splinted, zip-coded detection probes using antibodies

- Anti-PDGF-BB antibody covalently labeled via hydrazine linkages to a 15-base zip code sequence with a 20-base spacer of cytosine (C20).
- TPP reporter oligonucleotide with a complementary 15-base anti-zip code sequence linked via a C18 PEG spacer to a 10-base reporter sequence.
- AzC reporter oligonucleotide with an anti-zip code sequence and a 10-base reporter sequence complementary to the TPP reporter sequence. Anti-zip code sequence was 20 bases long, with 15 bases complementary to the zip code sequence.

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FIG. 16



Exemplary assembly of Antibody-linked, zip-coded Detection Conjugates.

- Primary amino groups in the antibodies are activated to hydrazines and reacted with 3' and 5'-aldehyde-containing oligonucleotides to form hydrazine linkages.
- The upper and lower two oligonucleotides linked to the antibodies also contain separate zip code sequences separated from the antibody by a C18 spacer arm.
- The inner two oligonucleotides (reporter oligonucleotides) consist of anti-zip code sequences linked through a C18 PEG spacer to a reporter oligonucleotide.
- One reporter oligonucleotide contains a 5'-terminal TPP residue, the other a 3' terminal AzC residue. Each zip code is complementary only to its anti-zip code, and the DPC reporter sequences are complementary only to each other.

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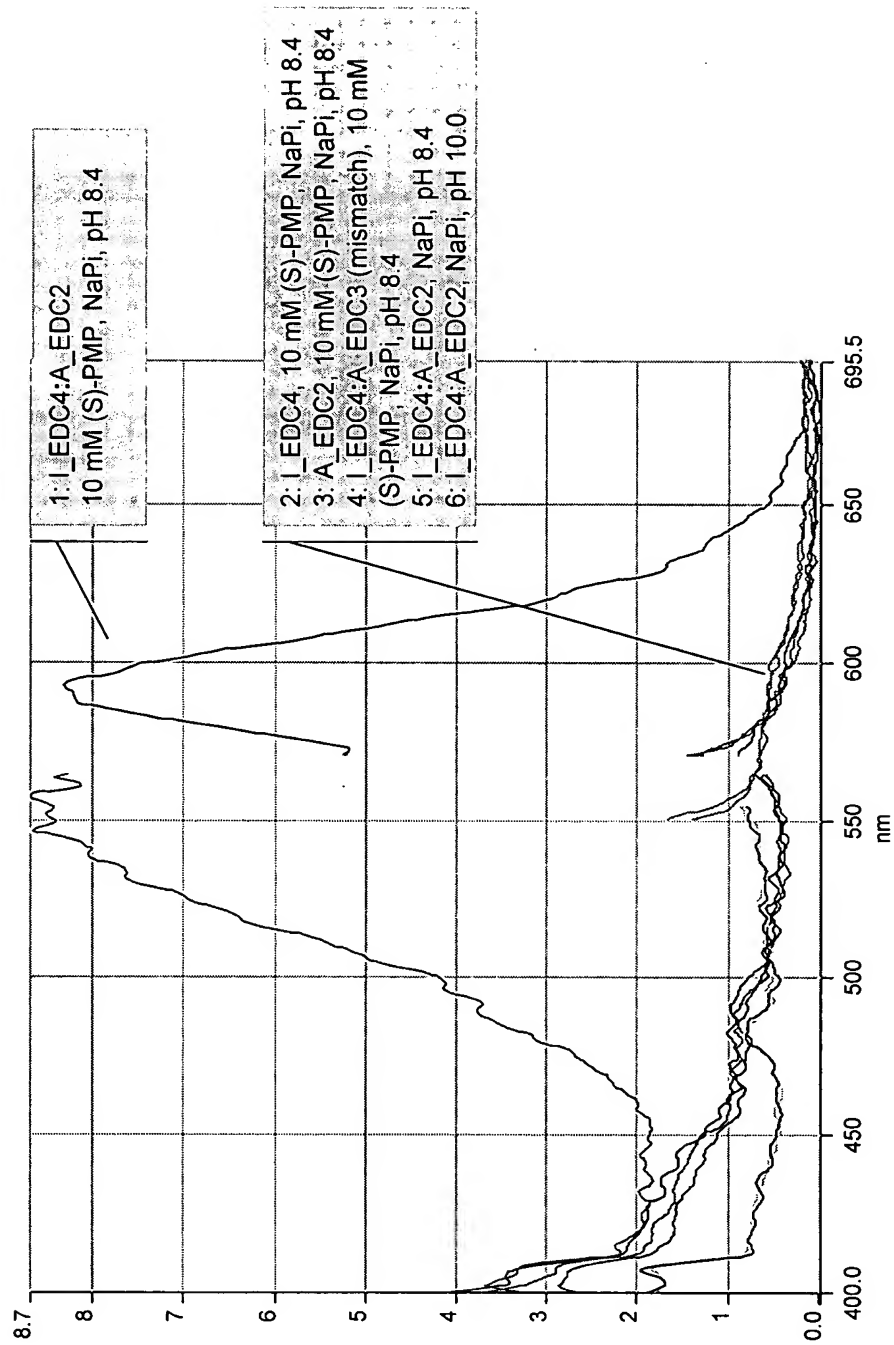


FIG. 17 Absorption and fluorescence emission spectra of DPC reaction mixtures (end of helix) after 2.3 hr at RT (200 nM each ssDNA, 1 M NaCl)

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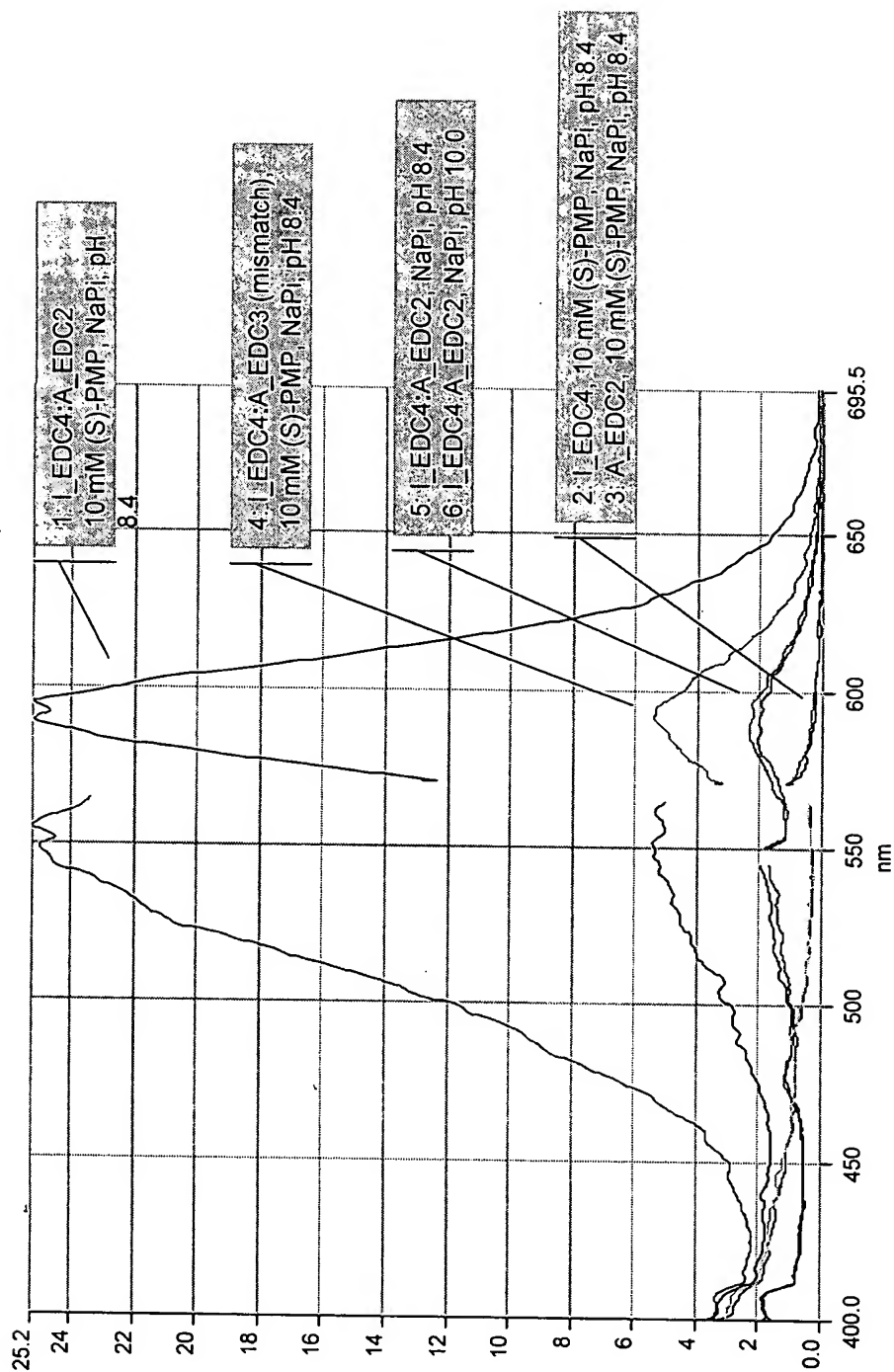


FIG. 18 Absorption and fluorescence emission spectra of DPC reaction mixtures (end of helix) after 16 hr at RT (200 nM each ssDNA, 1 M NaCl)

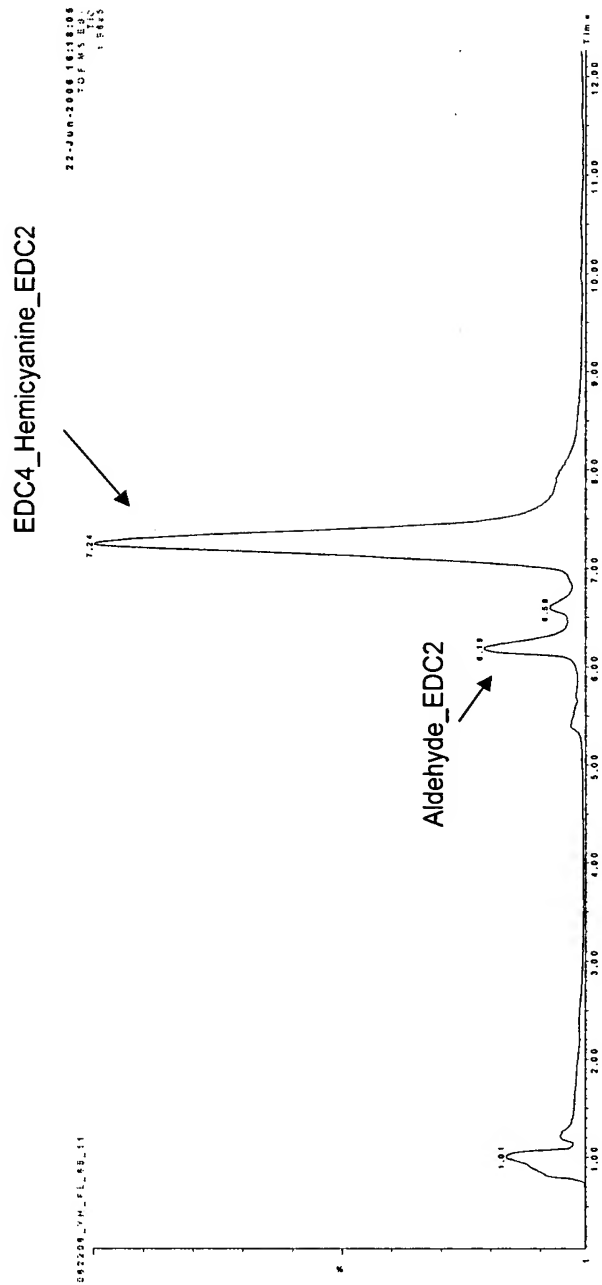


FIG. 19 LC-MS analysis of crude DPC reaction mixture of indolinium_EDC4:aldehyde_EDC2 after 16 hr at RT.

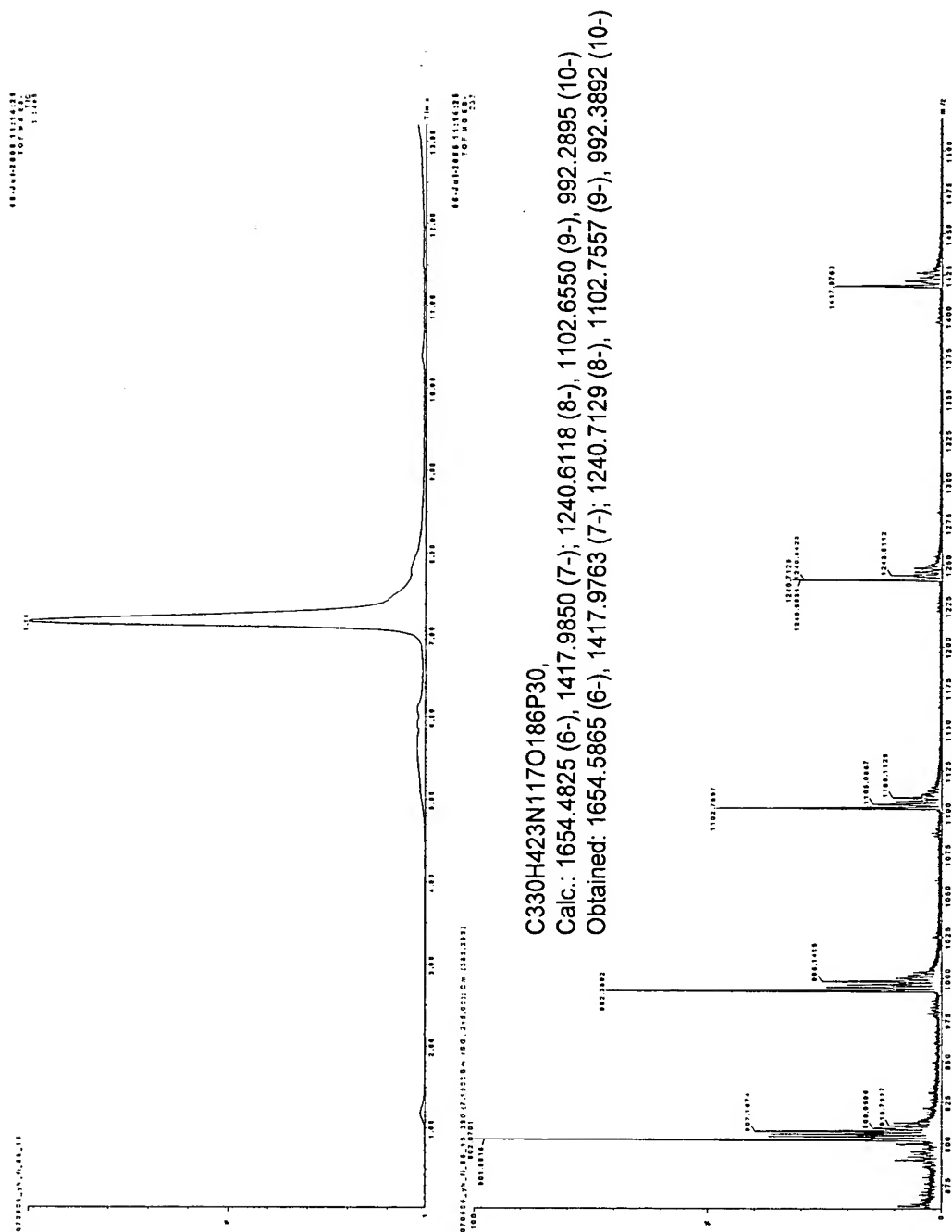


FIG. 20 LC-MS analysis of purified DPC product EDC4_Hemicyanine_EDC2.

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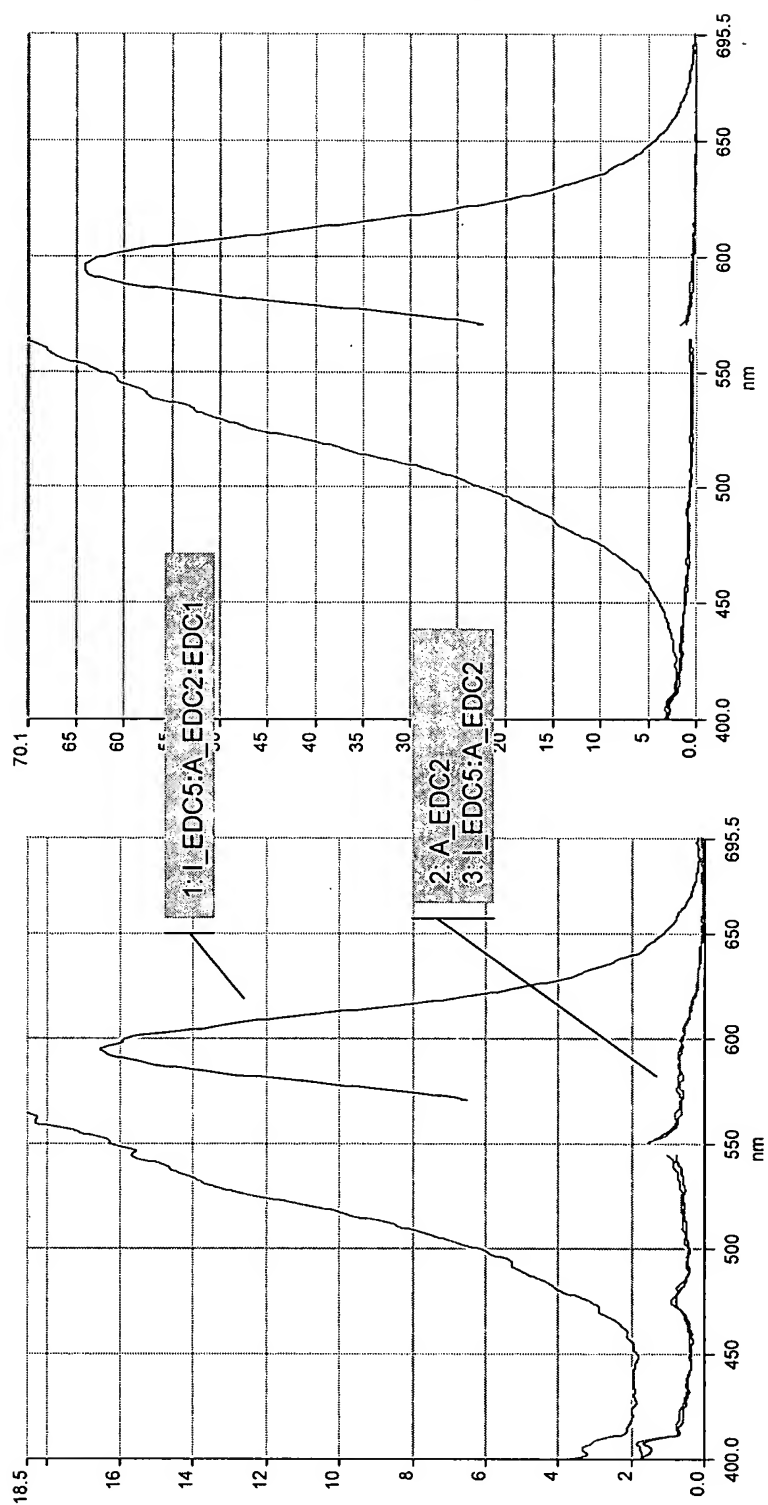


FIG. 21 Absorption and fluorescence emission spectra of DPC reaction mixtures (middle of helix) after 2.3 hr (left) and 16 hr (right) at RT (200 nM each ssDNA, 1 M NaCl, 50 mM NaPi, pH 8.4, 10 mM (S)-PMP)

C324H422N108O185P30
Calc.: 1942.5521 (-5), 1618.6267 (-6), 1387.2515 (-7), 1213.7200 (-8); 1078.7511 (-9); 970.7760 (-10).
Obtained 1942.5461 (-5), 1618.6136 (-6), 1387.3574 (-7), 1213.8362 (-8); 1078.7533 (-9); 970.8626 (-10).

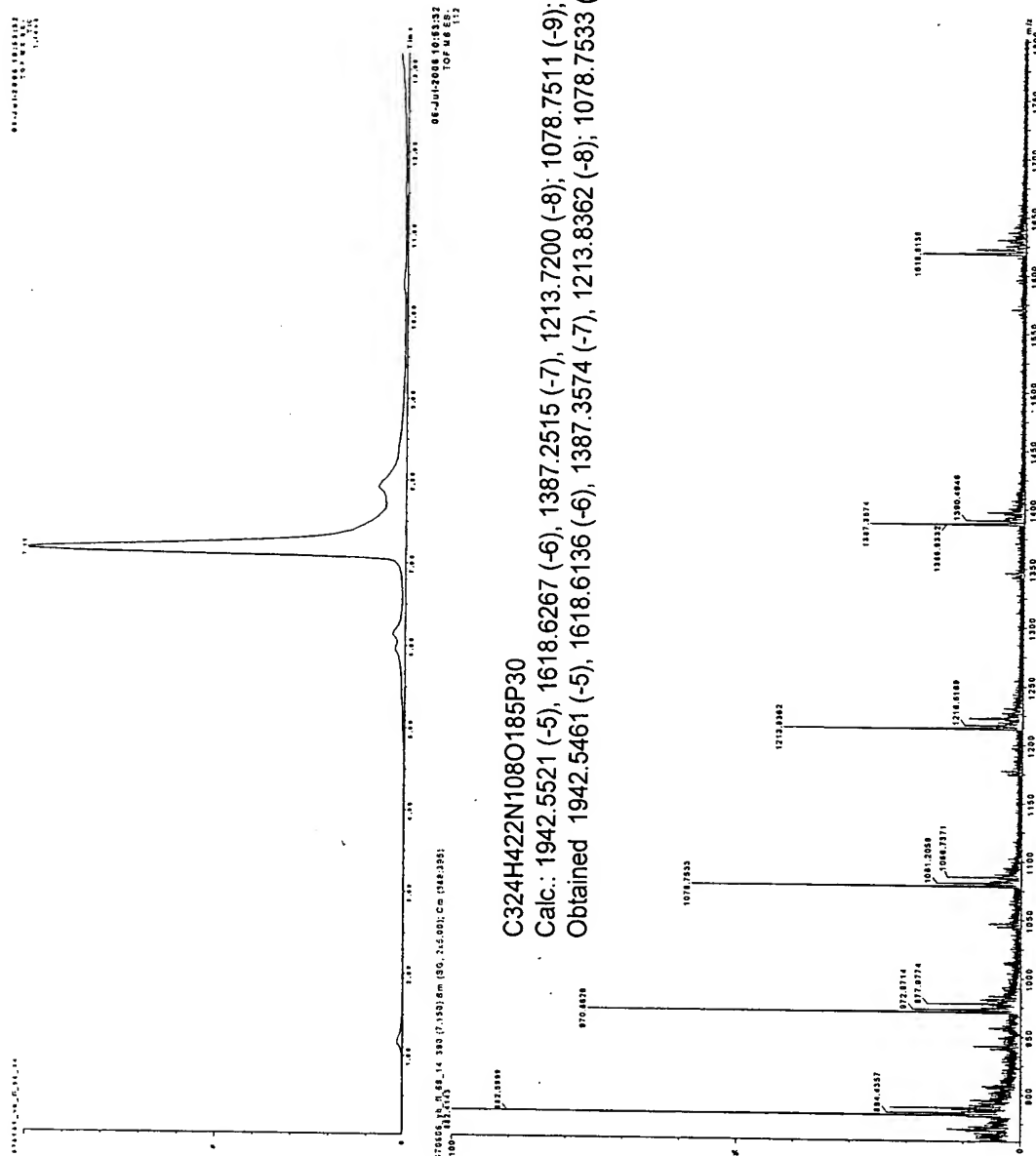


FIG. 22 LC-MS analysis of purified DPC product EDC5_Hemicyanine_EDC2.

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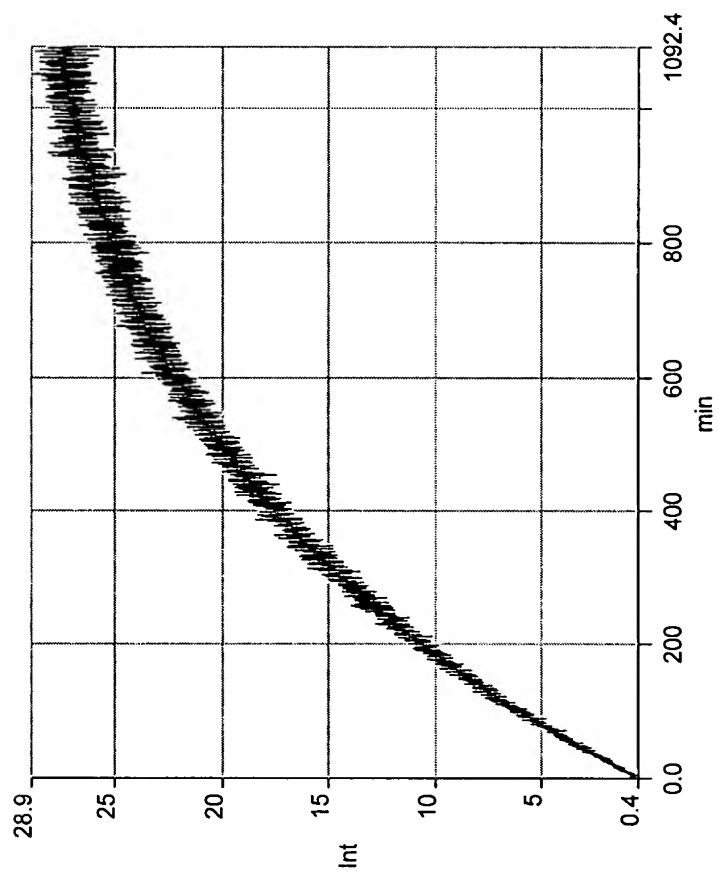


FIG. 23 Plot of fluorescence intensity at 590 nm vs time for DPC of Indolinium_EDC4 and Aldehyde_EDC2 (Excitation at 550 nm)

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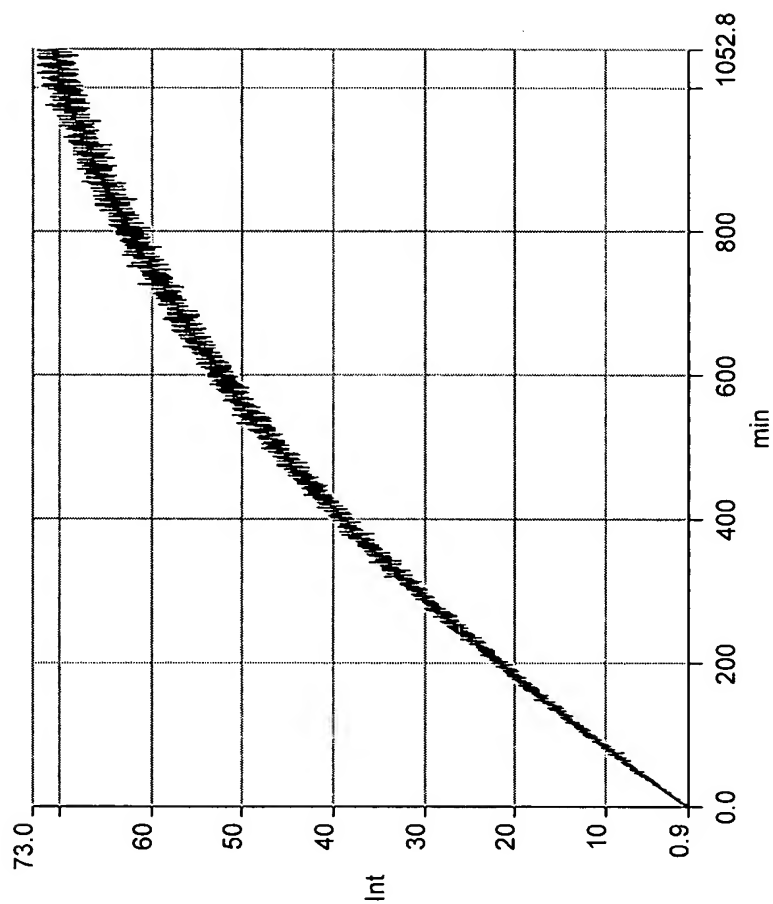


FIG. 24 Plot of fluorescence intensity at 590 nm vs time for DPC of Indolinium_EDC5, EDC1 and Aldehyde_EDC2 (Excitation at 550 nm).

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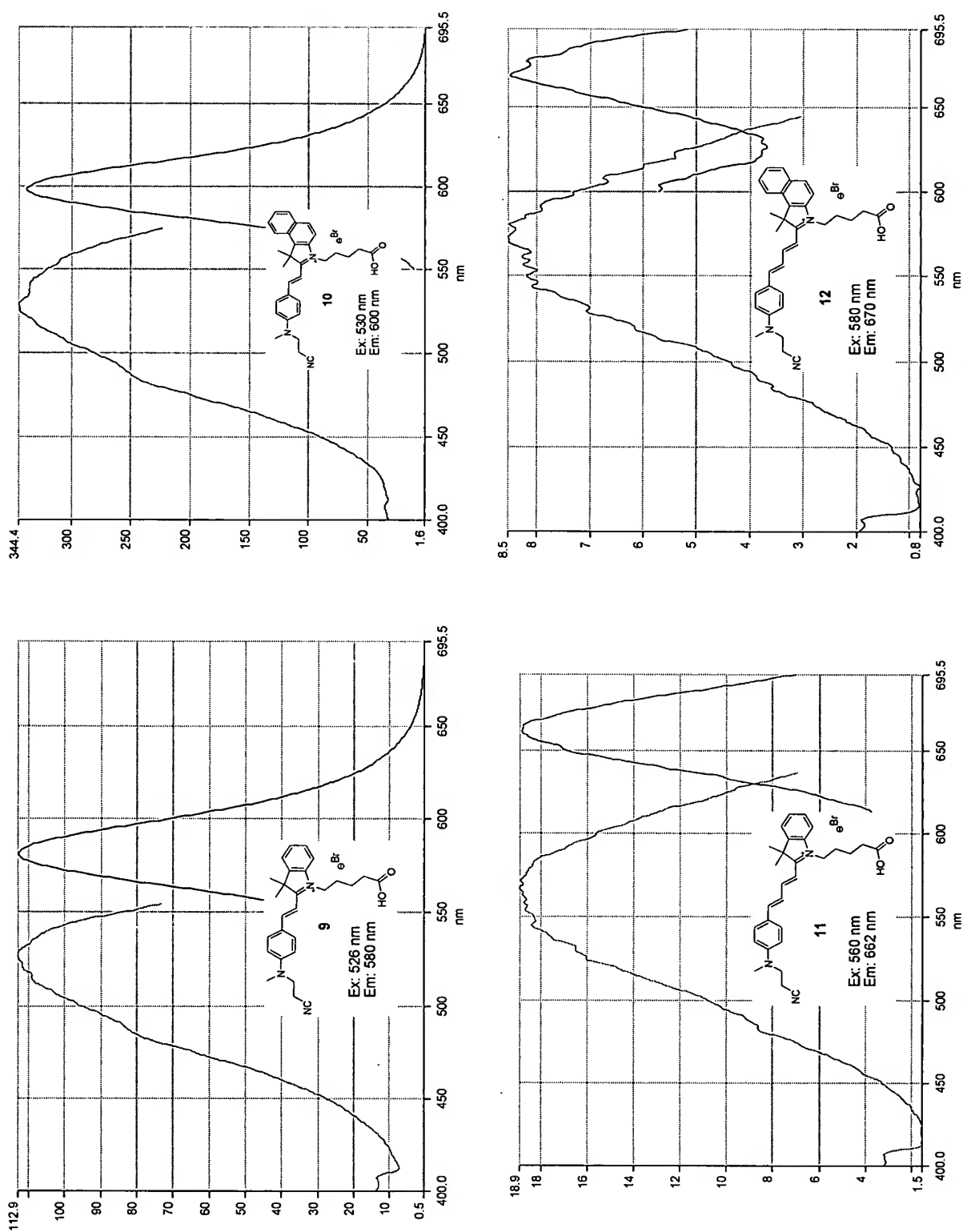


FIG. 25 Fluorescence excitation and emission spectra of four hemicyanine dyes.

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Sequence Name	EDC#	Sequence	[Seq]	Units	Function	structure
(693-687-688-493)	EDC1	GTGGTAGTTGGAGCTGGTGGCGTAGGCAAGA (SEQ ID NO. 74)	200	nM	Target	I_H1_A0
(693-687-56-16)	EDC10	GTGGTAGTTGGAGCTGGAGCGGCAACGGAGA (SEQ ID NO. 81)	200	nM	Target	I_H2_A1
(37-101-688-493)	EDC11	GACGTCTTCAAGGGTGGTGGCGTAGGCAAG (SEQ ID NO. 82)	200	nM	Target	BI_H1_A0
(37-101-56-16)	EDC12	GACGTCTTCAAGGGTGGAGCGGCAACGGAGA (SEQ ID NO. 83)	200	nM	Target	BI_H2_A1
(693-687)	EDC2	*AGTCCAACTACCAC (SEQ ID NO. 75)	600	nM	Probe	Indolinium (I)
(688-493)	EDC5	TCTTGCCCTACGCCAC* (SEQ ID NO. 78)	400	nM	Probe	Aldehyde0 (A0)
(37-101)	EDC7	*ACCCTTGAACACGTC (SEQ ID NO. 79)	600	nM	Probe	Benzoinindolinium (BI)
(56-16)	EDC8	TCTCCGTGGCCGCTC* (SEQ ID NO. 80)	400	nM	Probe	Aldehyde1 (A1)

FIG. 26 DNA sequences for four-plex hemicyanine dye generation.

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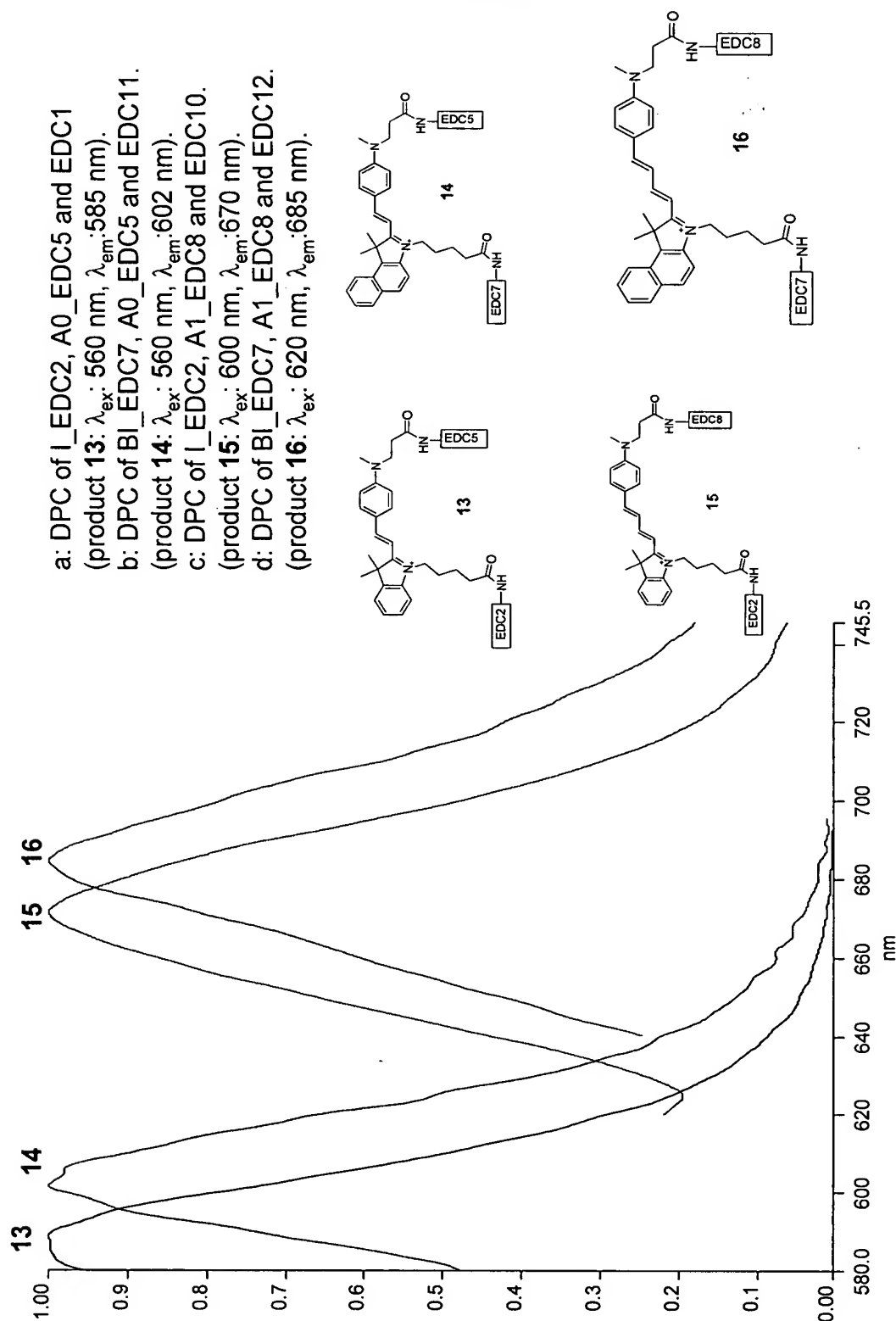


FIG. 27 Normalized fluorescence spectra of four individual DPC reactions (a to d) after 140 minutes at RT (the chemical structures of the DPC products **13** to **16** are shown on the right).

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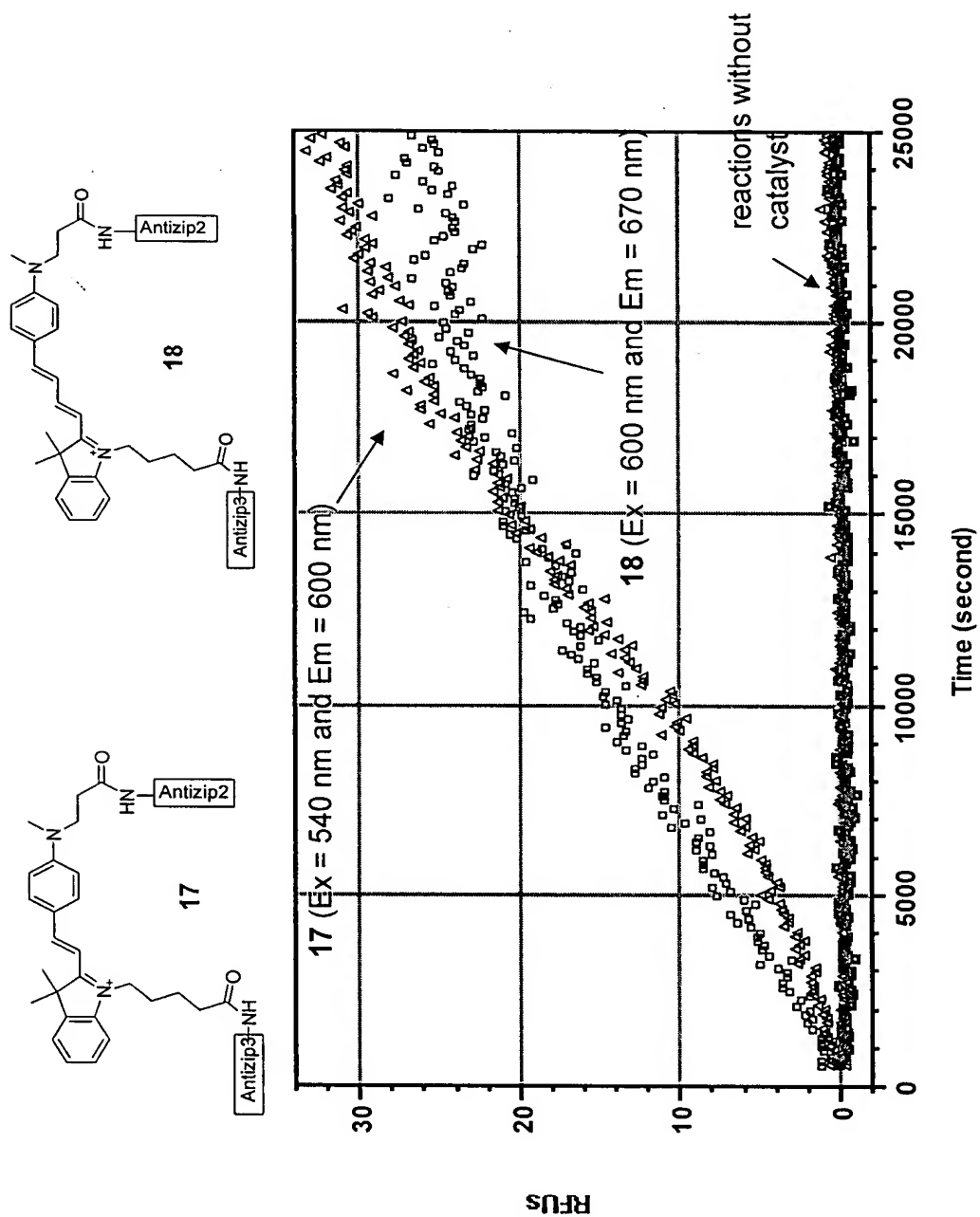
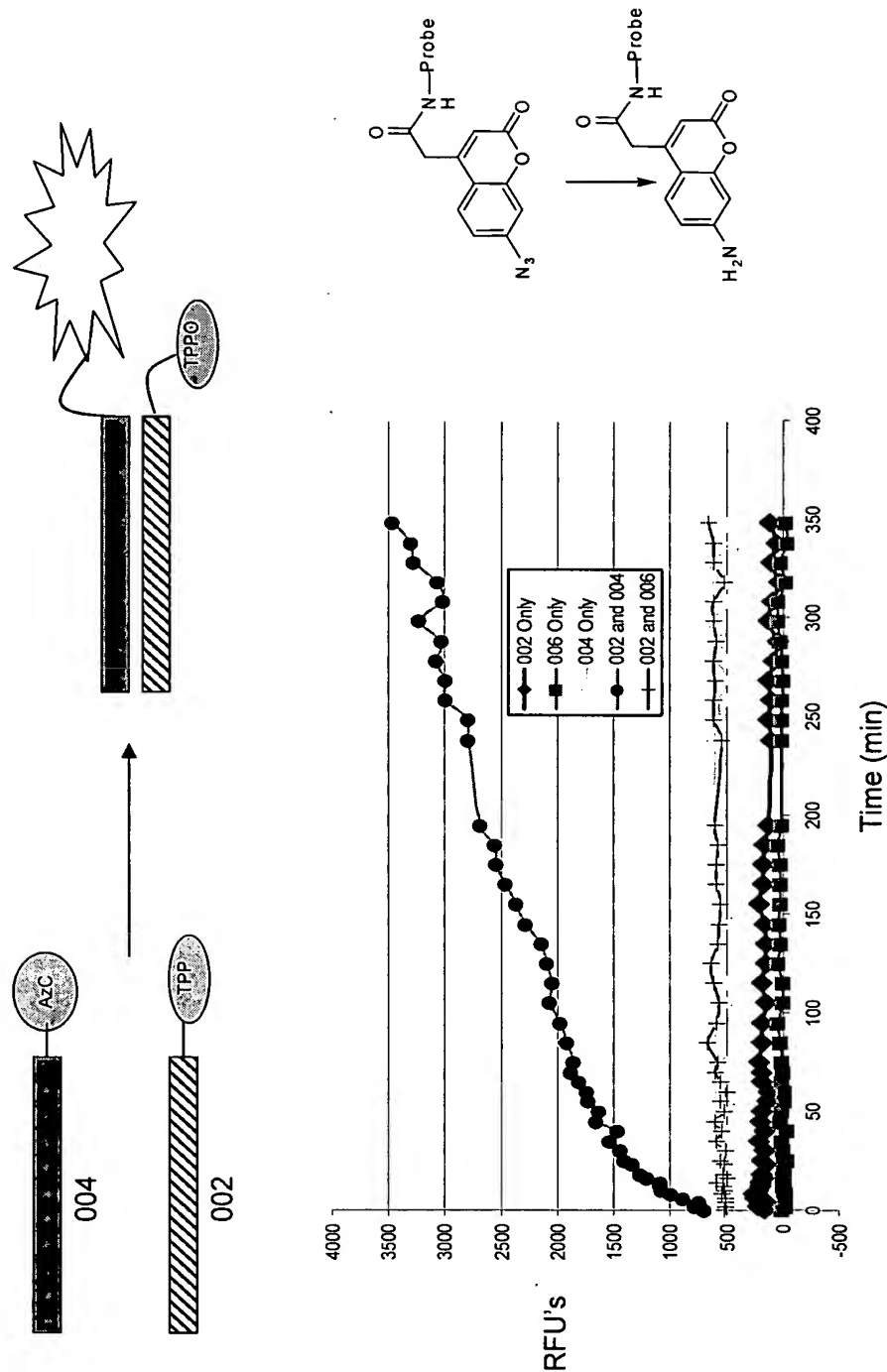


FIG. 28 Fluorescence kinetic analysis of DPC reactions of Antizip3_indolinium/Antizip2 reporter1_A0 (triangle) and Antizip3_indolinium/ Antizip2 reporter1_A1 (circle).

FIG. 29



RFU = Relative Fluorescent Units

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FIG. 30

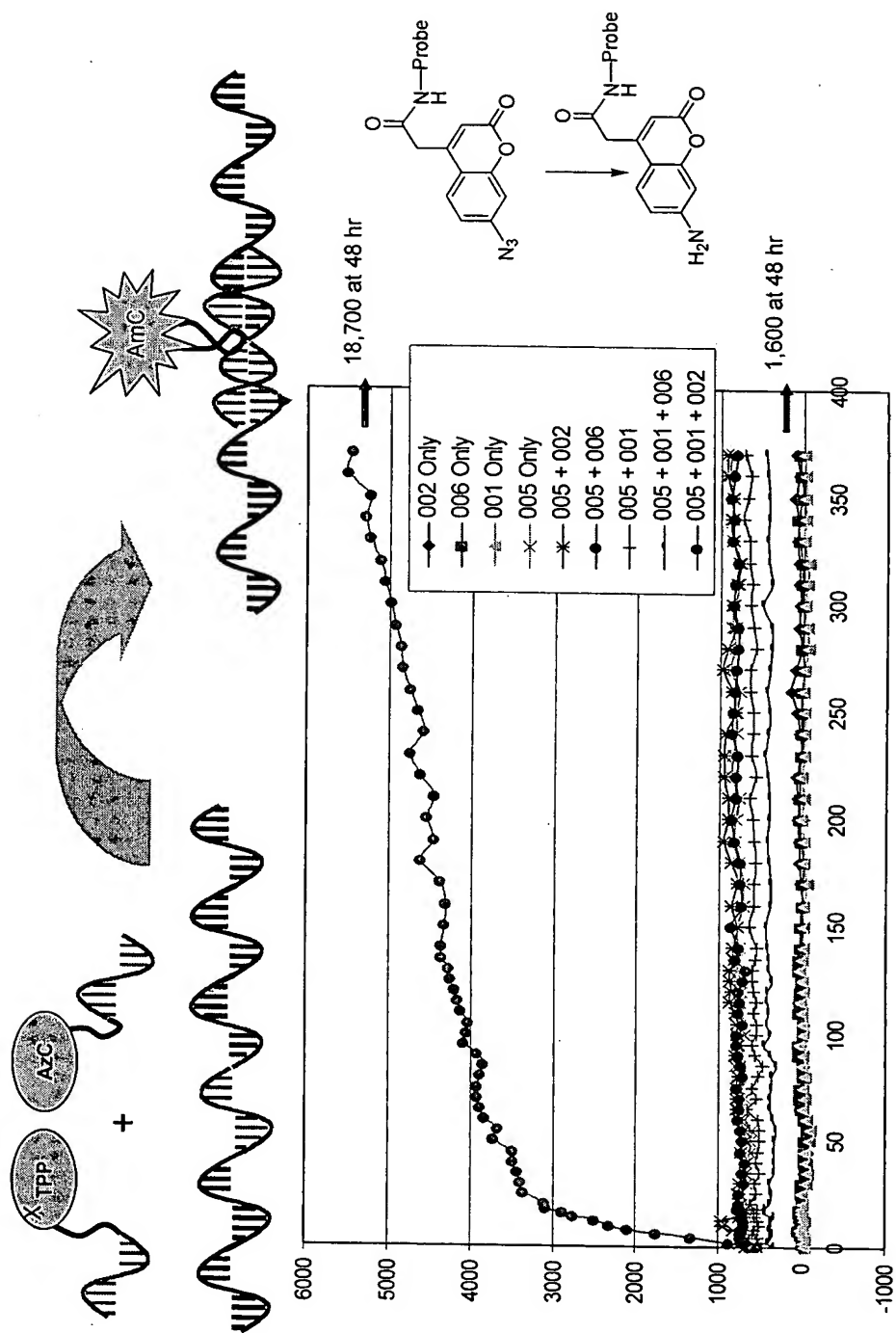
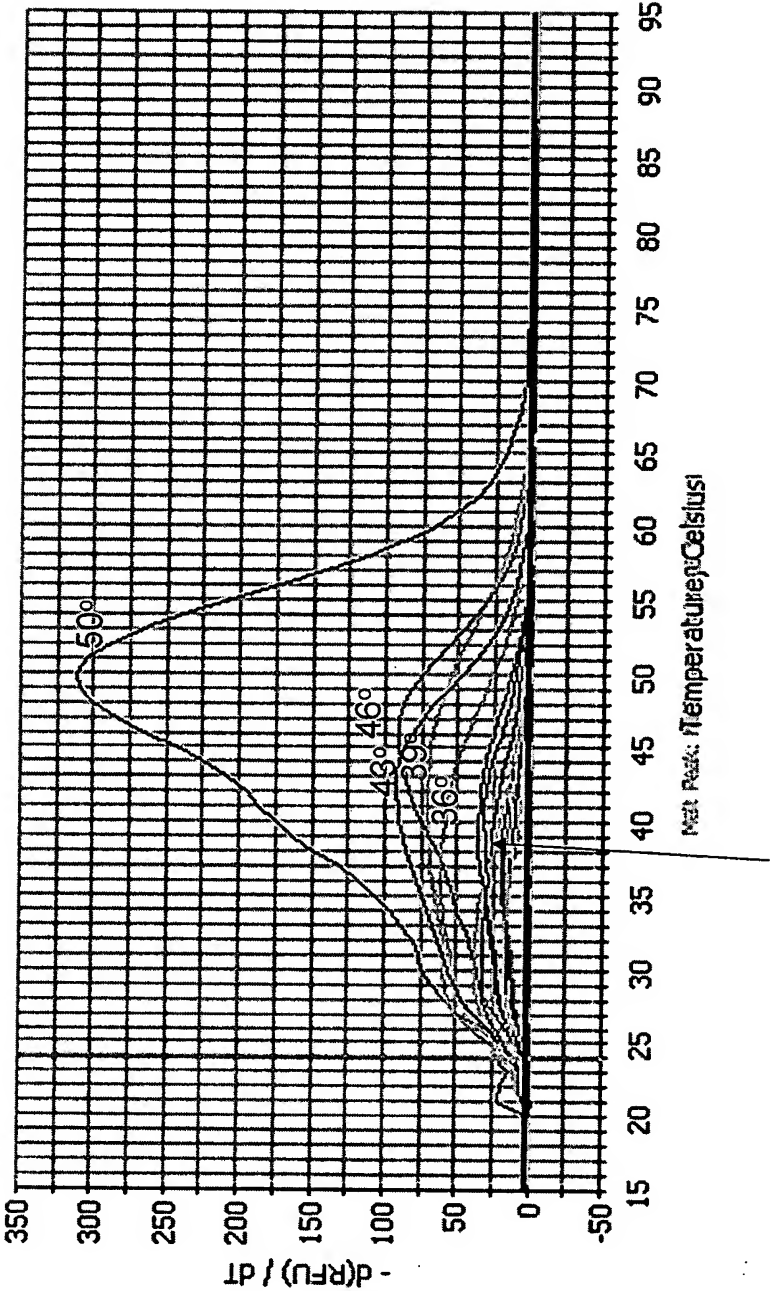


FIG. 31

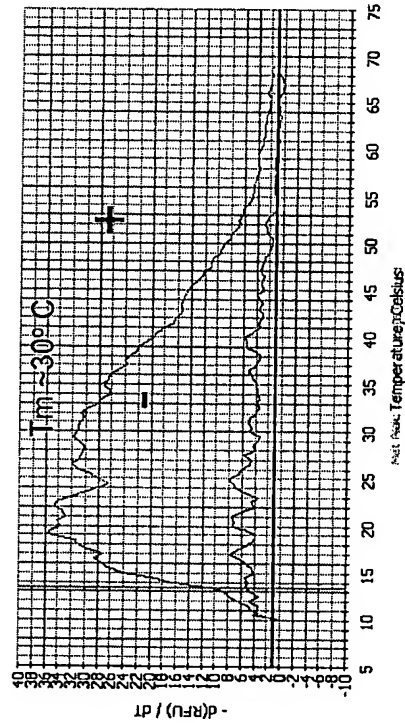
Melt Curves, 10-base Complementary Oligos
1.0 M NaCl, 500 nM -20 nM Oligos



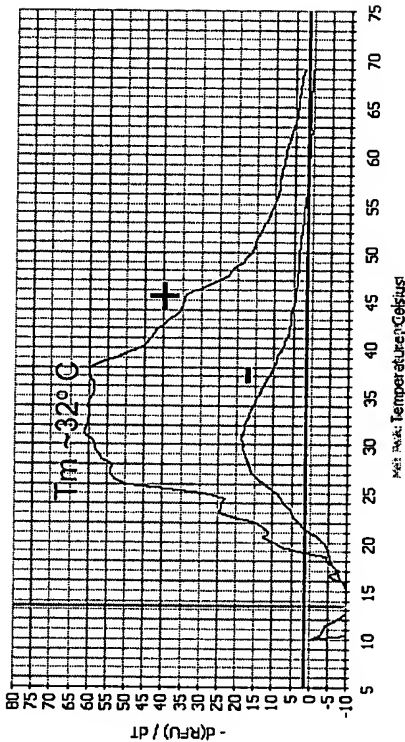
2-base mismatches – T_m 's 42° C to 31° C
Drop in $T_m \sim 10^\circ$ C per 10-fold concentration of oligos

FIG. 32

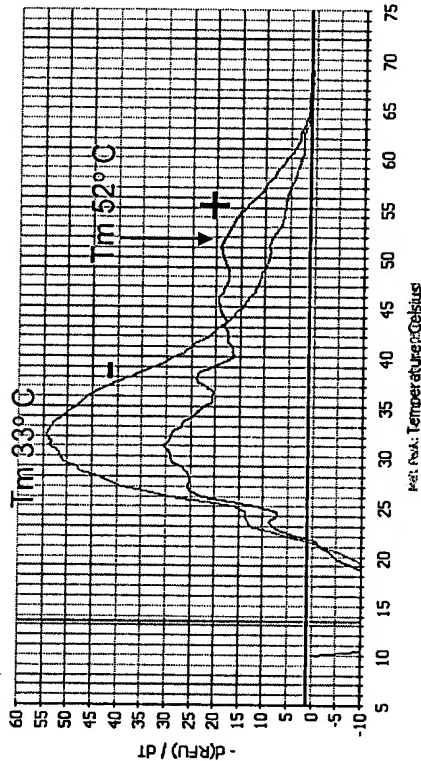
Non-biotinylated oligo 23



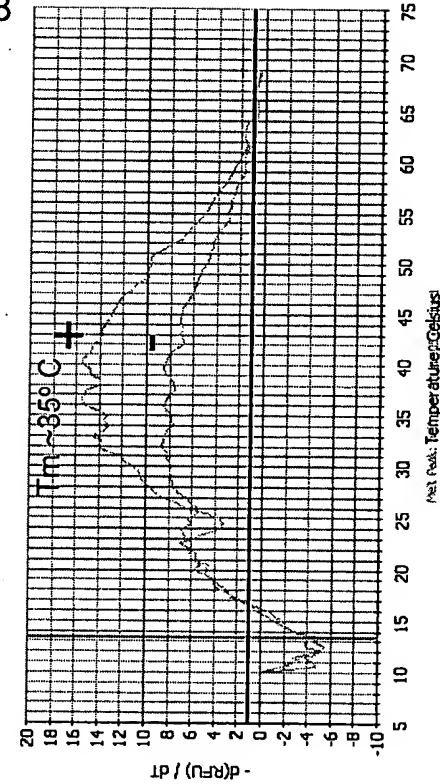
Complementary non-biotinylated oligos



Complementary biotinylated oligos



Partially complementary biotinylated oligos

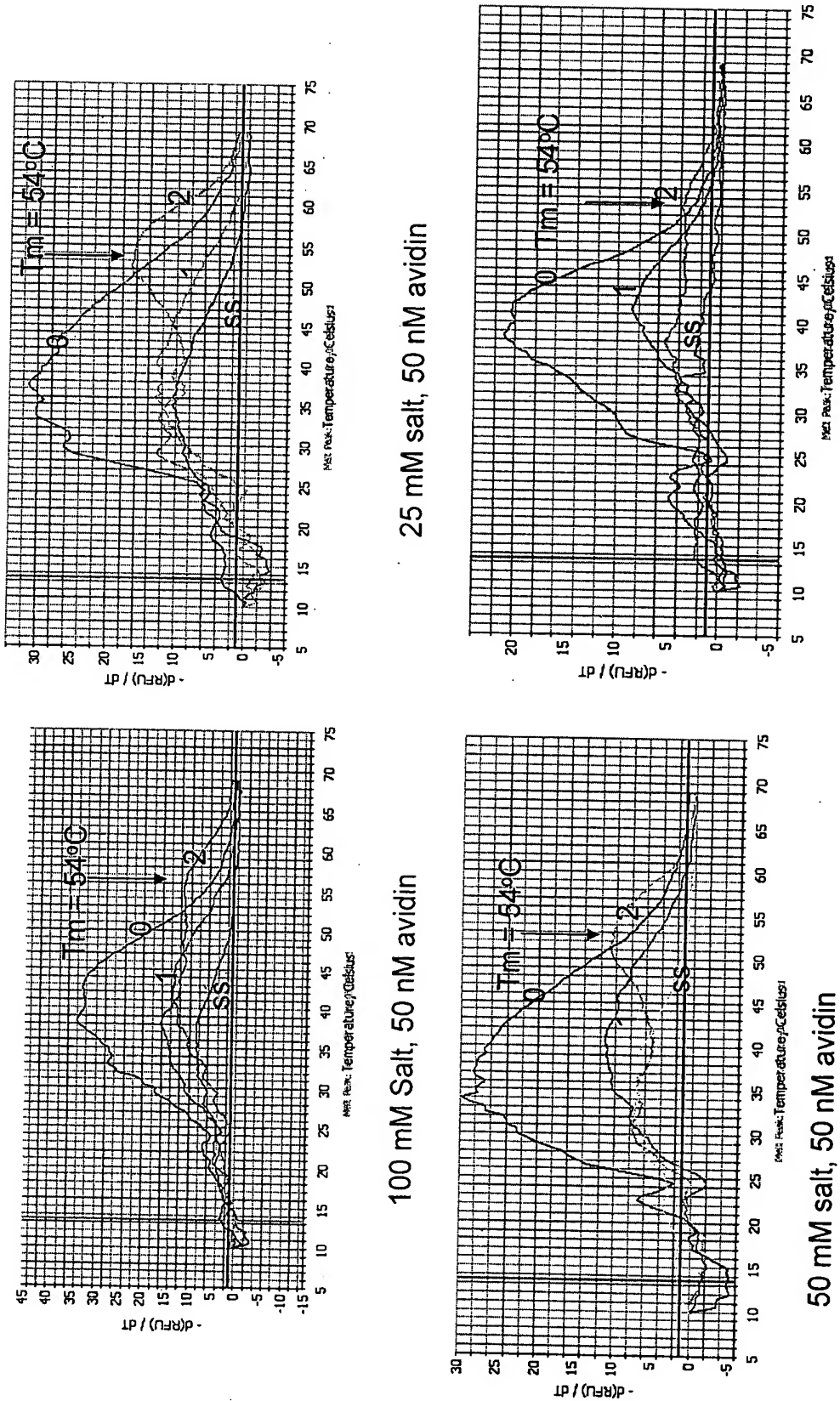


"Hot Start" DNA Hybrid Melting Curves +/- Avidin
100 nM oligos, 100 nM avidin, 25 mM salt

FIG. 33

Complementary Biotinylated Oligos

T_m Increases from ~35°C to ~54°C Upon Binding to Avidin.



2= both strands biotinylated, 1= 1 strand of two biotinylated 0 = both strands not biotinylated ss= one biotinylated strand only

FIG. 34

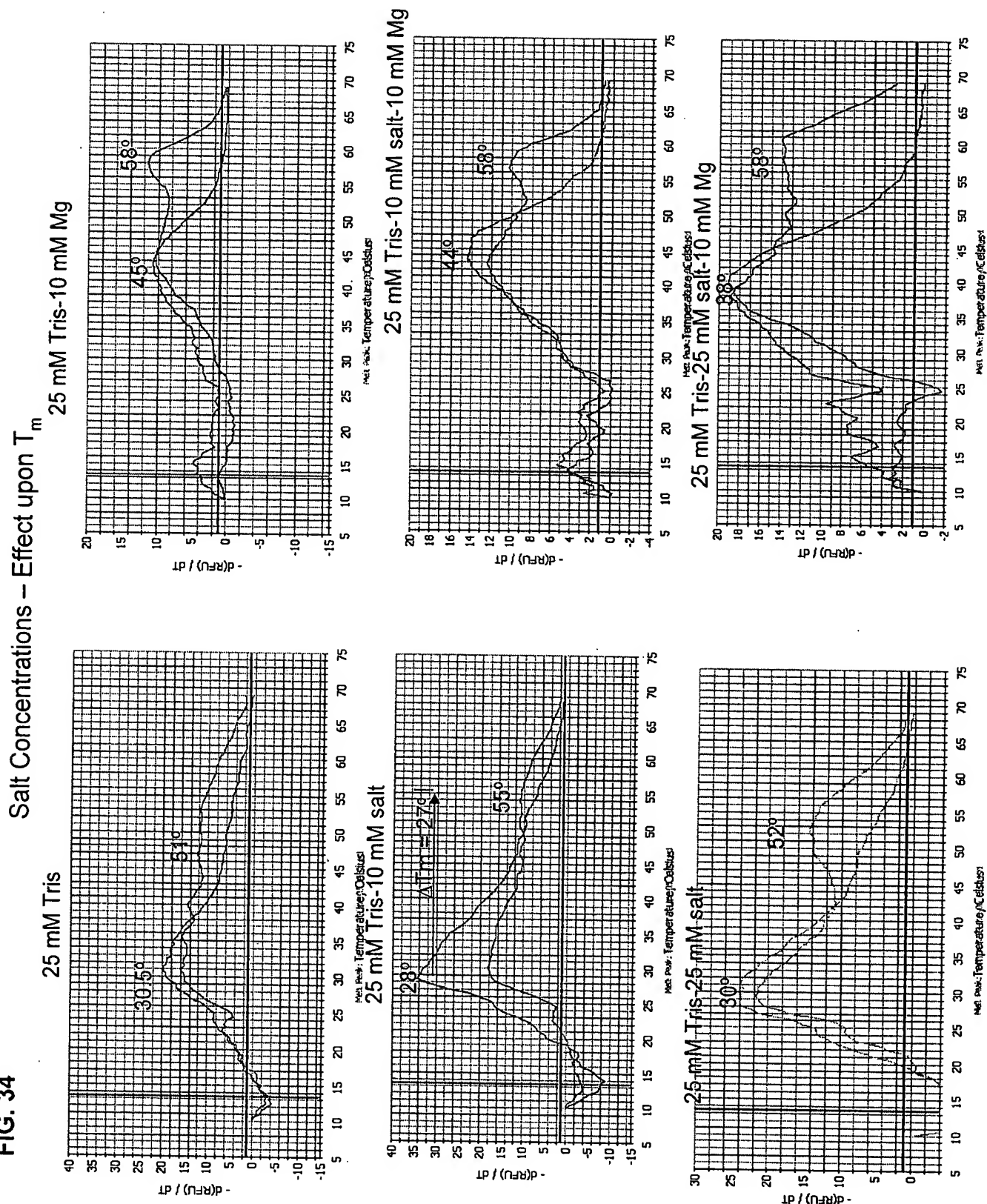
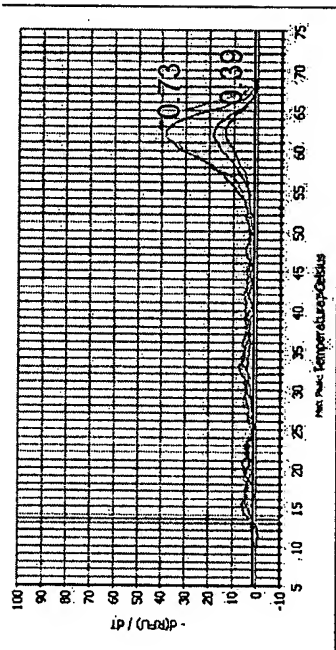
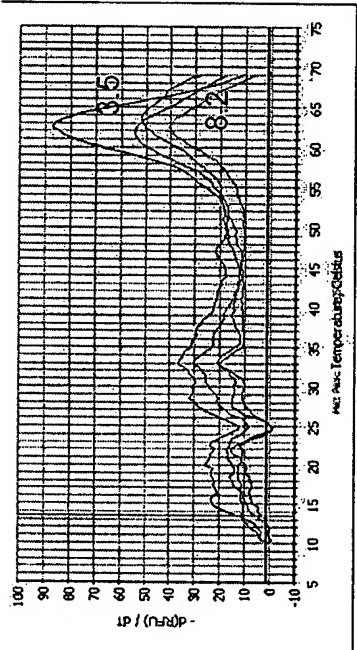
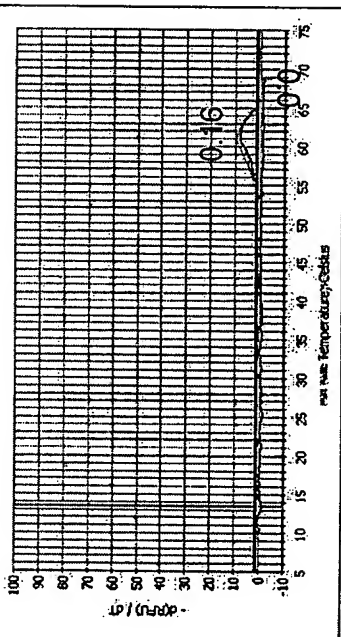
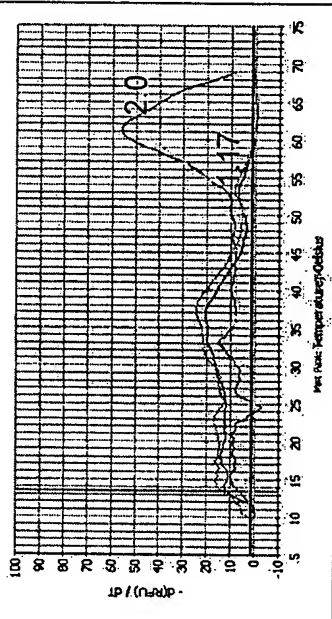
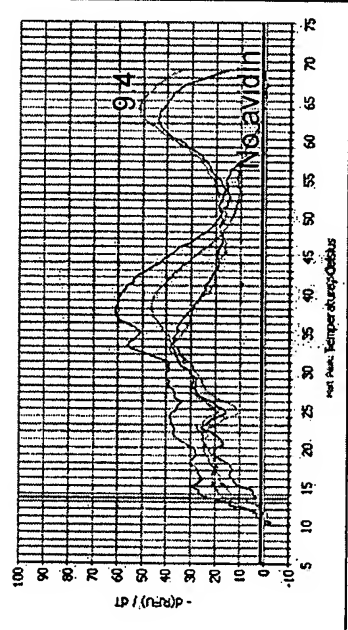


FIG. 35

Ratios of Biotinylated Oligos to Avidin



1st derivative of Melting Curves for
A-T rich biotinylated oligos
Molar ratio of oligos to Avidin
Oligos + avidin = constant of 7 μ M

FIG. 36

Melting Curves of 5' and 3' (-) Biotin-Strand Oligos duplexed with Biotin-5' (+) Strand Oligo

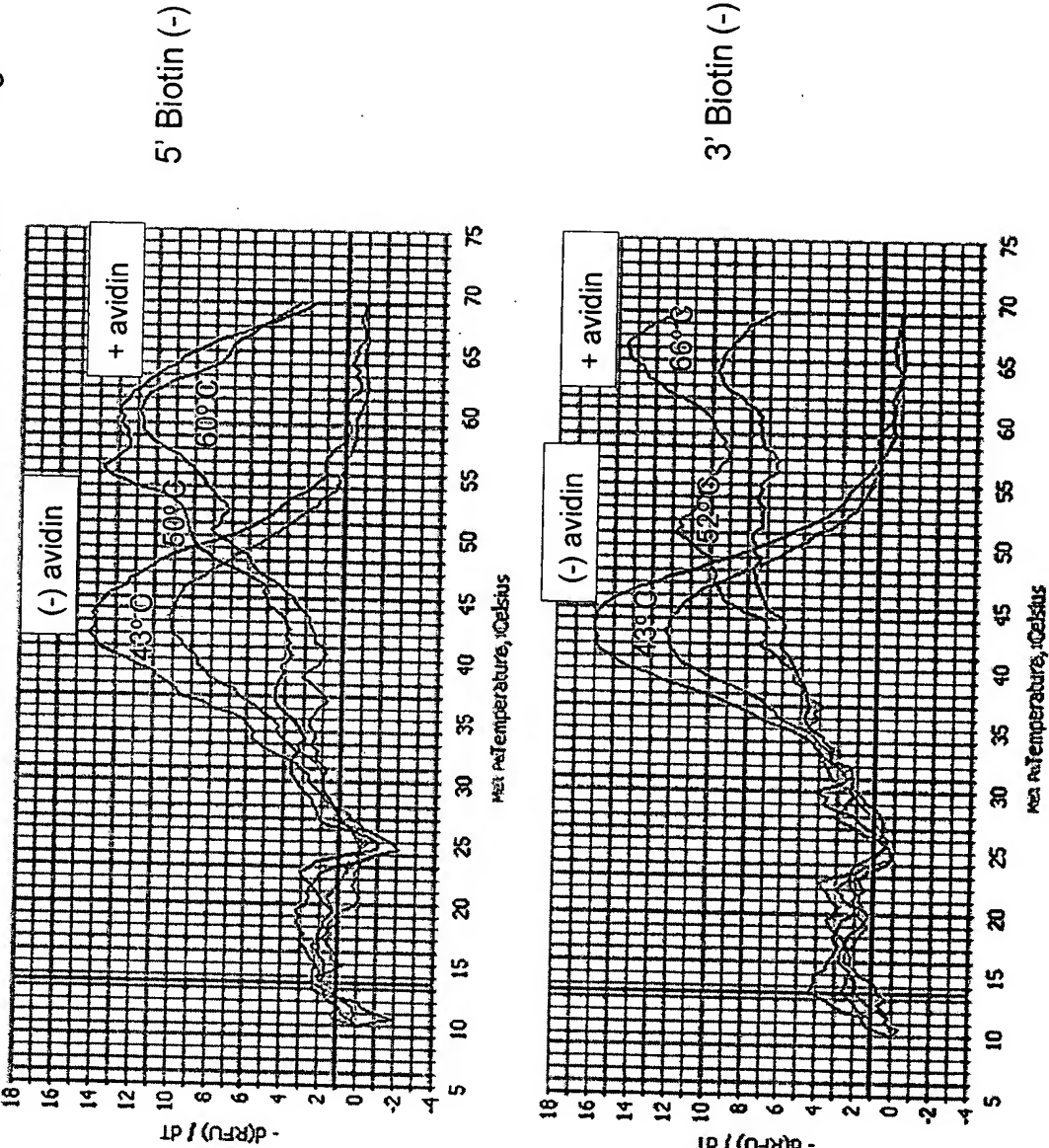
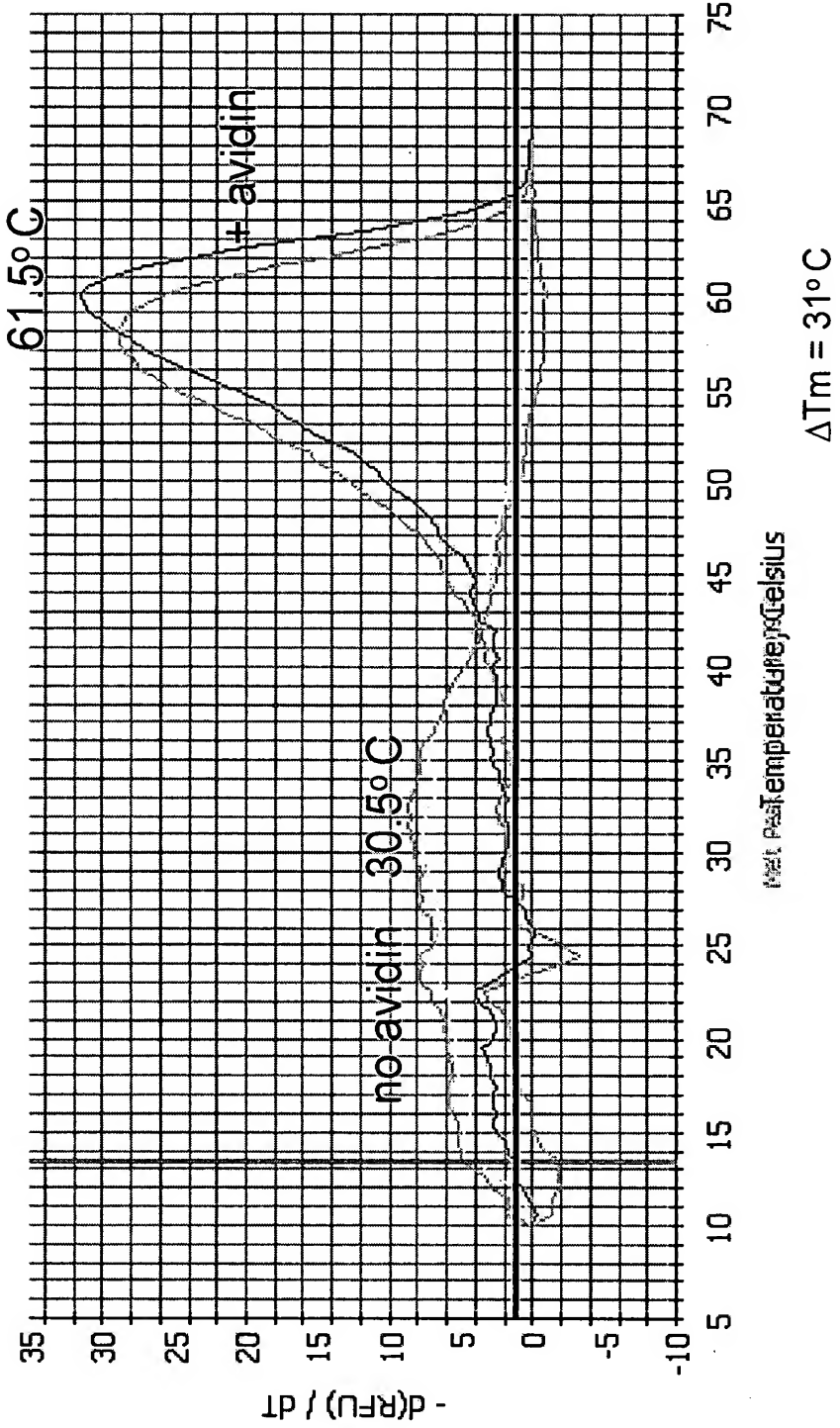


FIG. 37
Melting Curves of AT-rich Biotinylated Oligo
Dimers +/- Target

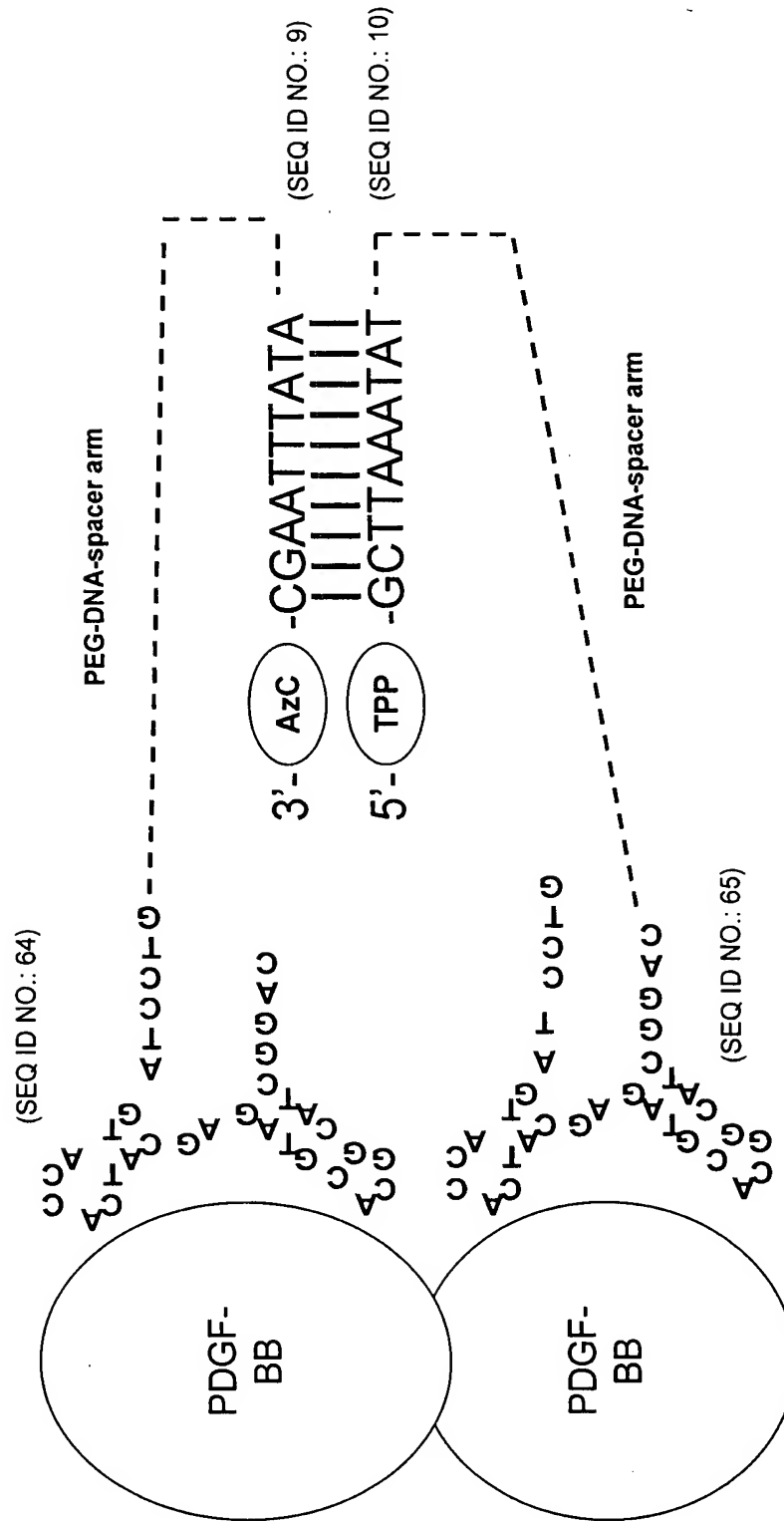


(+) 5' -TTTTTTTTTTTTTAAATAAA-3' (SEQ ID NO: 27)
(-) 3' AAATTAATTTTTTTTTTTT-5' (SEQ ID NO: 27)

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FIG. 38

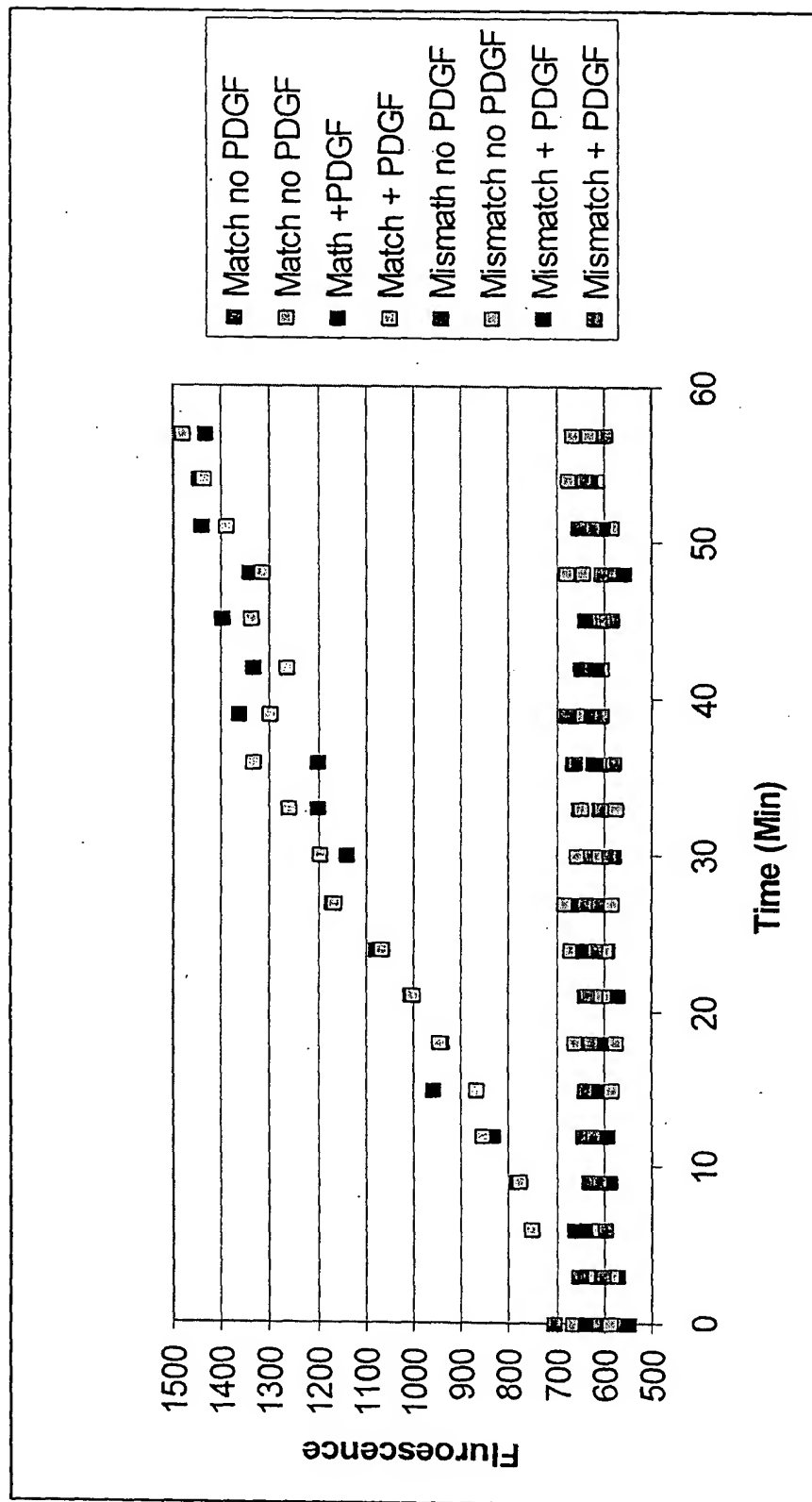
Detection of PDGF-BB with Aptamer-DPC Oligonucleotides



- Aptamers to B-type subunits of PDGF are conjugated to two different, complementary oligonucleotides through a single stranded DNA-PEG spacer arm.
- The azidocoumarin and triphenylphosphine precursors are in a helical structure only when the T_m is raised by the aptamers binding to PDGF.

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FIG. 39

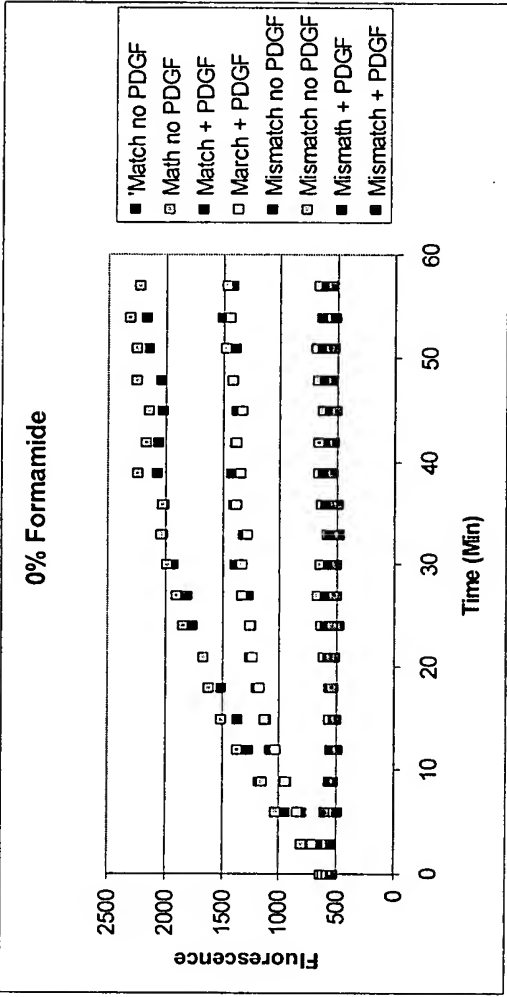


Reaction of the aptamer probes to PDGF does not occur

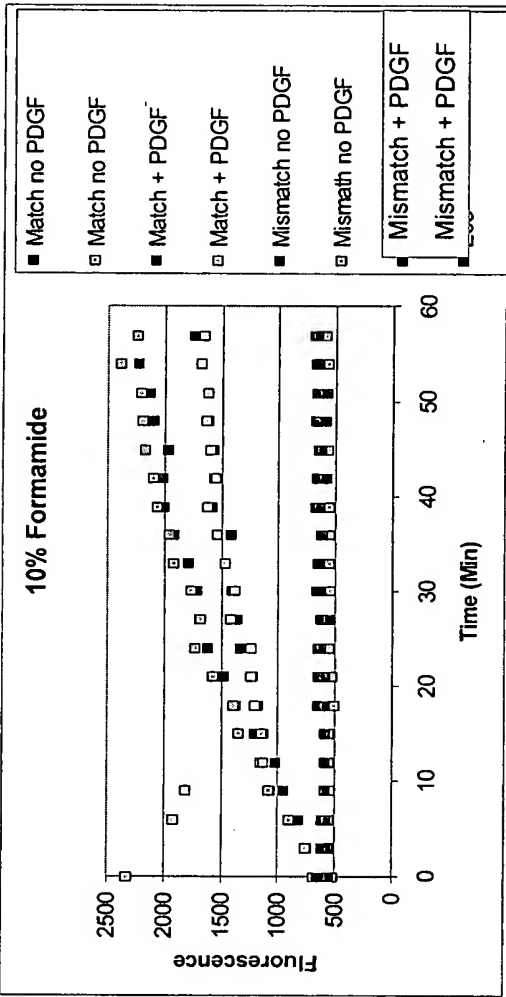
- 1) in the absence of PDGF
- 2) if the TPP probe is mismatched in DNA sequence to the Coumarin probe.

FIG. 40

Rates in Formamide



a)

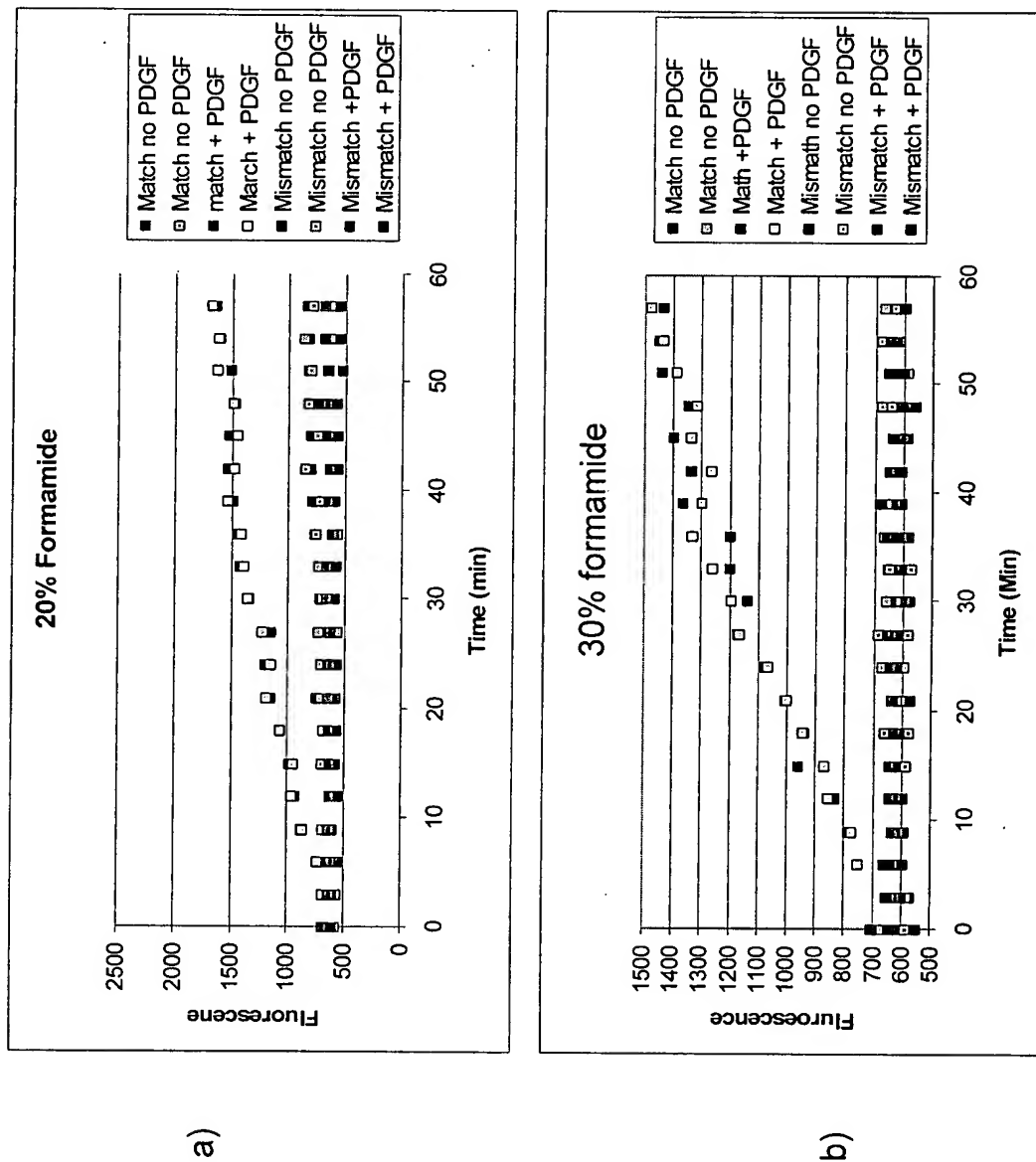


b)

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FIG. 41

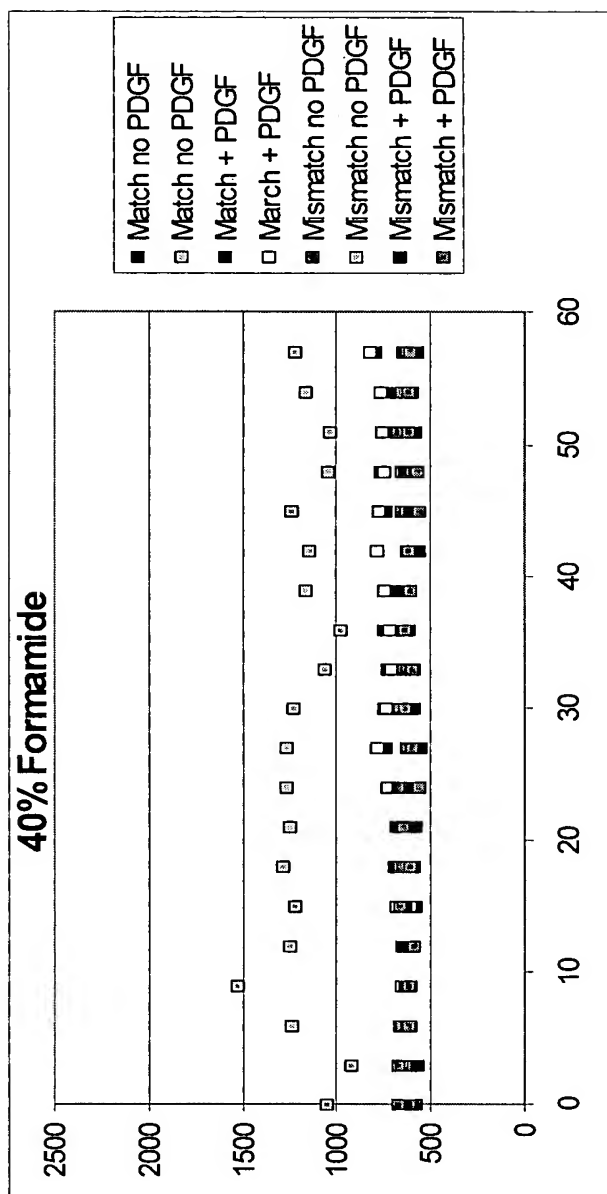
Rates in Formamide



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FIG. 42

Rates in Formamide



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FIG. 43

Homogeneous Assay Detection of PDGF-BB with Aptamer-based Probes

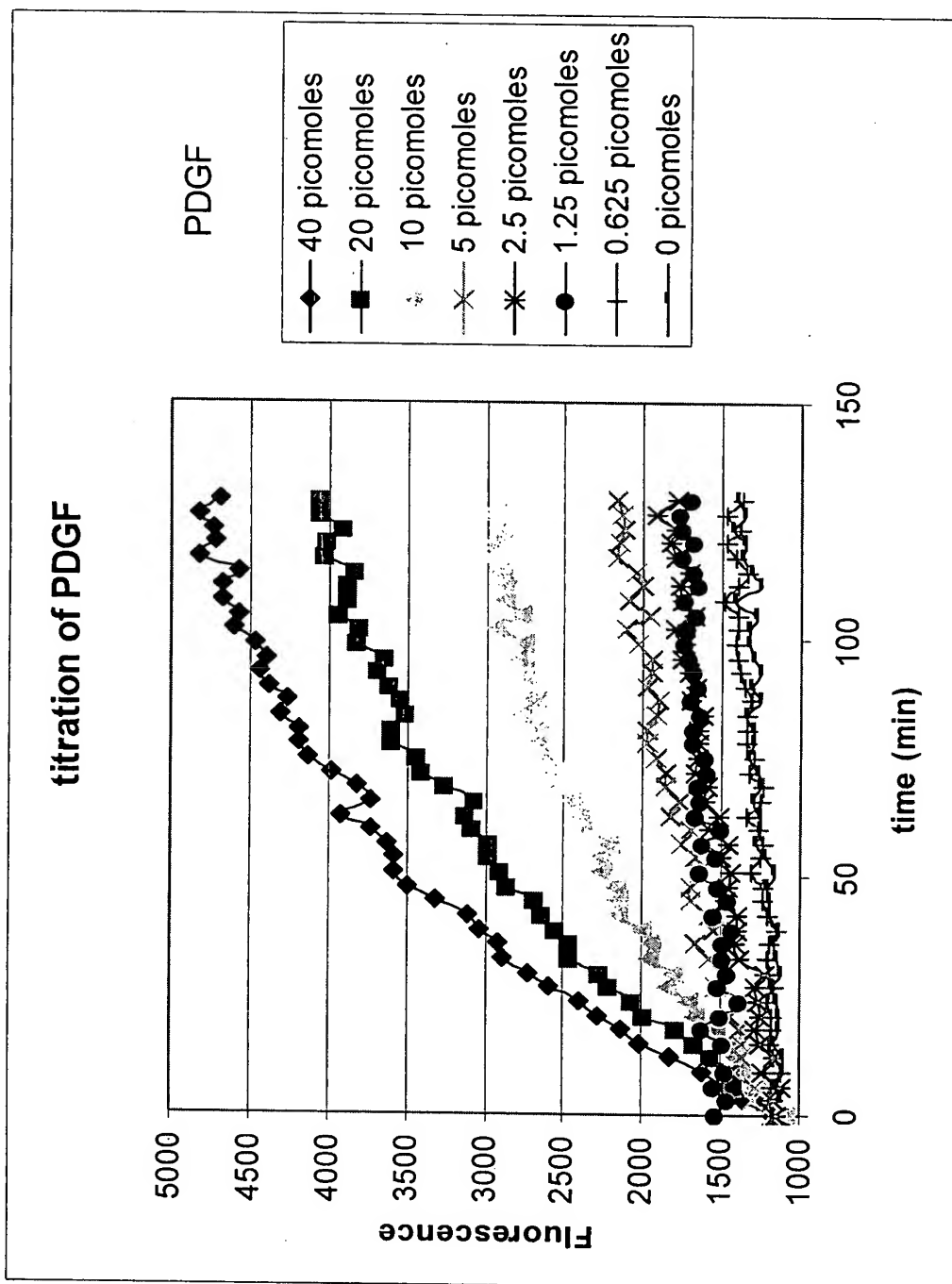


FIG. 44
Homogeneous Assay Detection of PDGF-AA with Aptamer-DPC Probes

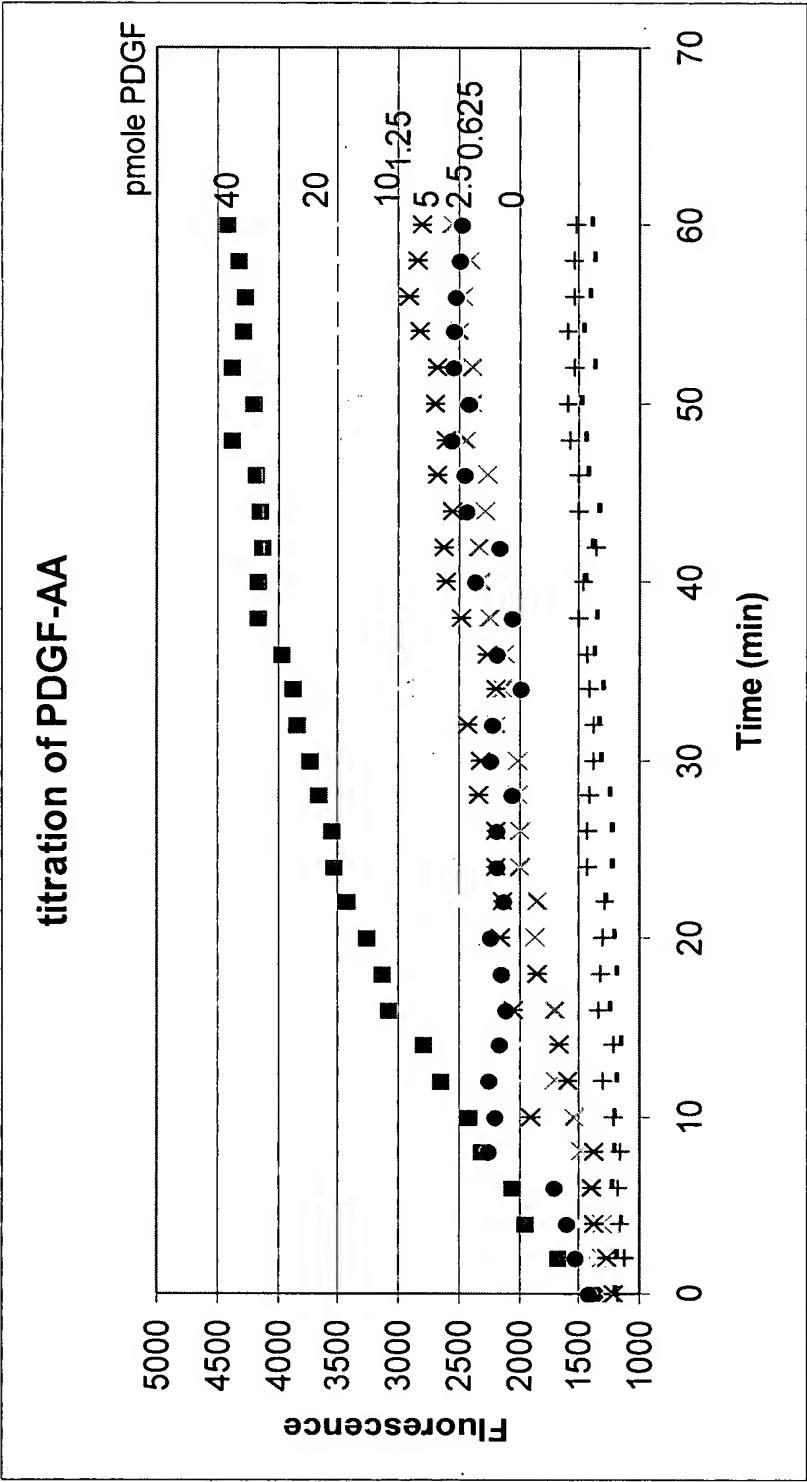
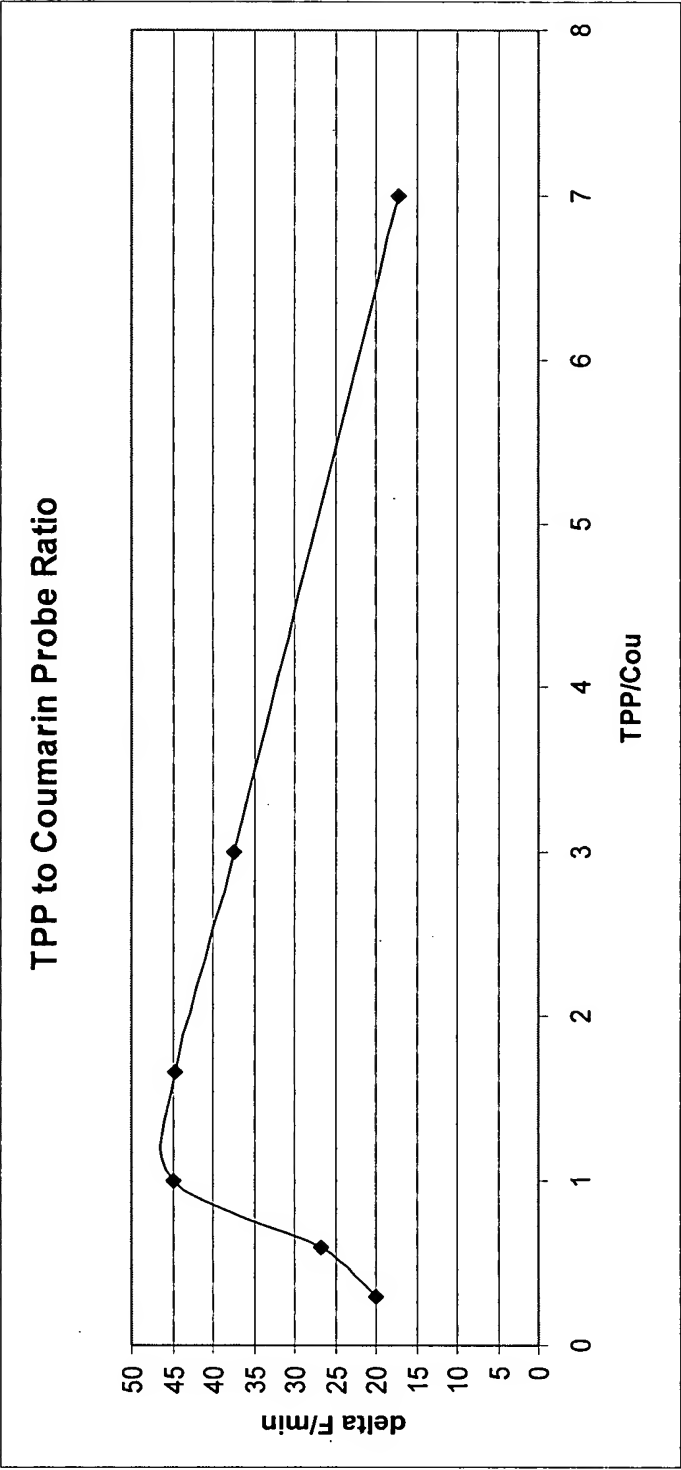


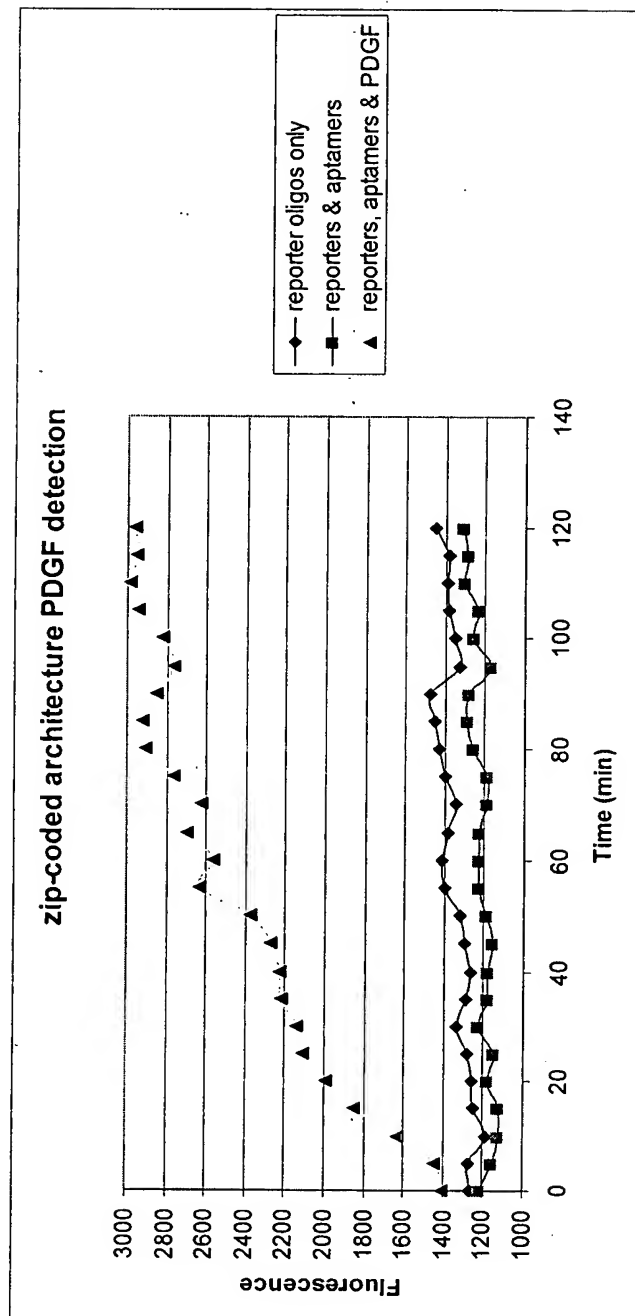
FIG. 45

Ratio of TPP:AZC Probes



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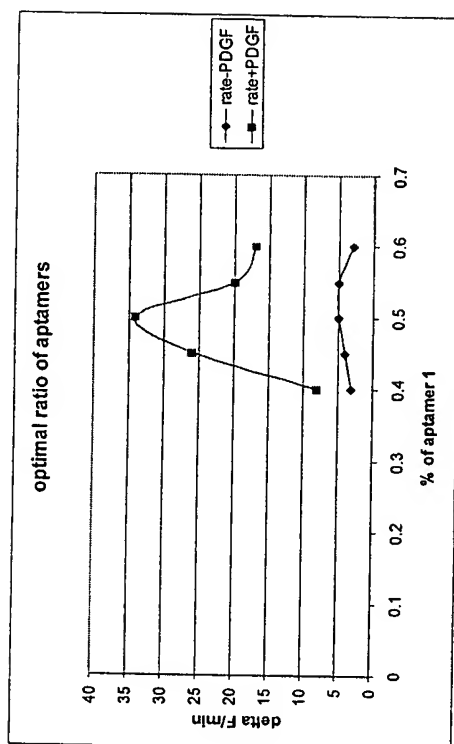
FIG. 46



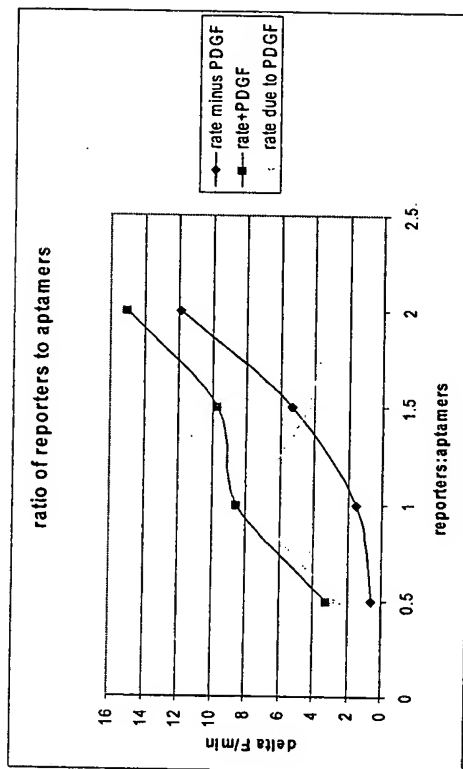
- Dependence of the splinted architecture upon the presence of both aptamer and reporter oligonucleotides.
- All reactants were tested at 0 or 0.4 μM concentration, at 22°C, in 50 mM Tris/HCl pH 8.5 – 10 mM MgCl_2 – 35% v/v formamide.
- Fluorescence was measured in a Wallac Victor Luminometer with excitation at 350 nm and emission at 460 nm.

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FIG. 47



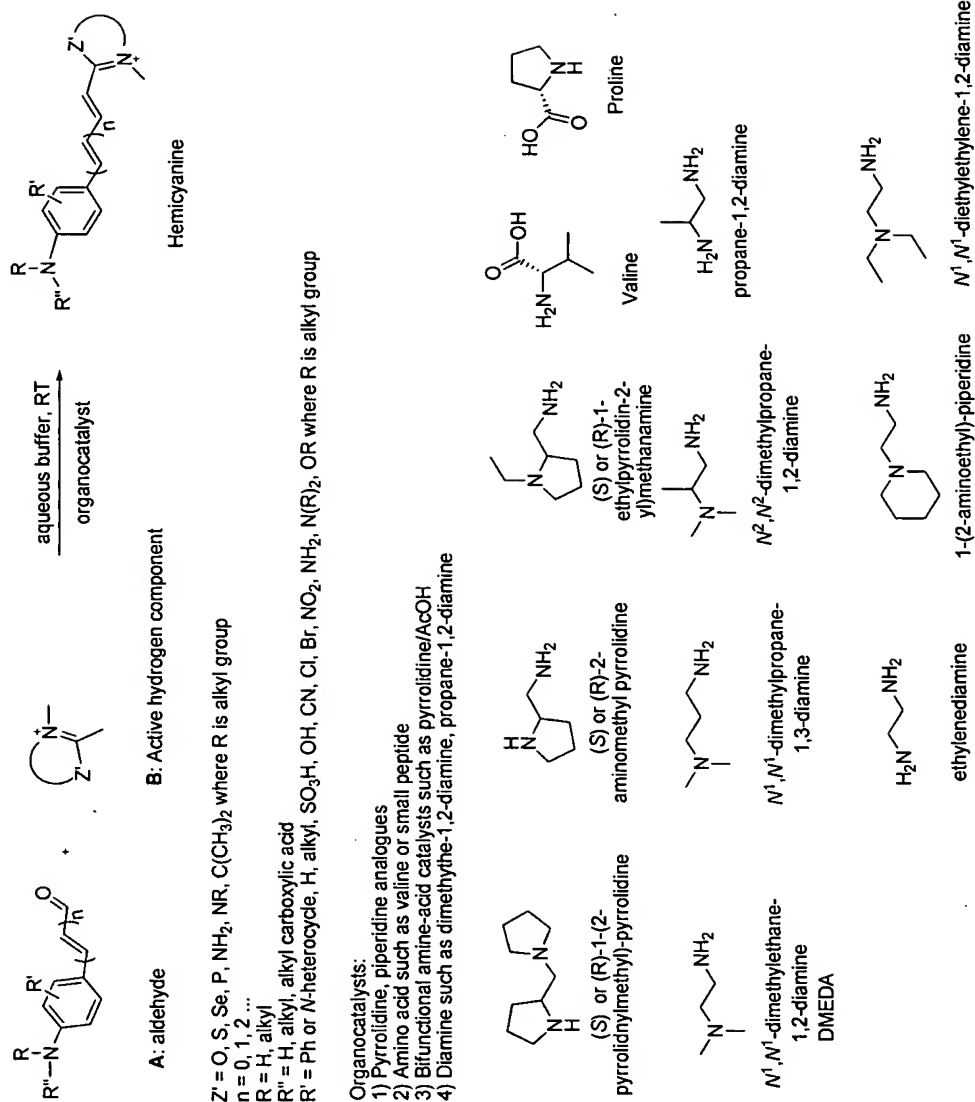
A)



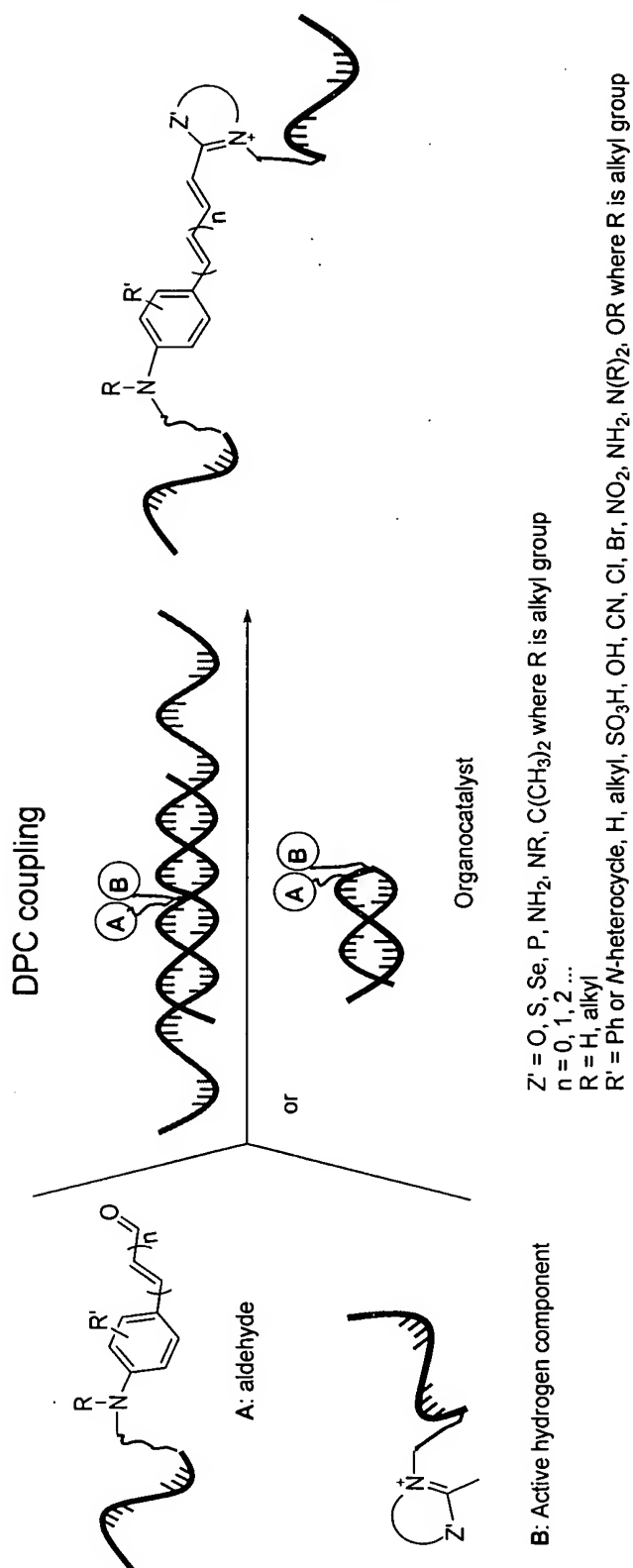
B)

Reaction rates at various ratios of TPP to AzC aptamers oligonucleotides (A) or reporters to aptamers (B).

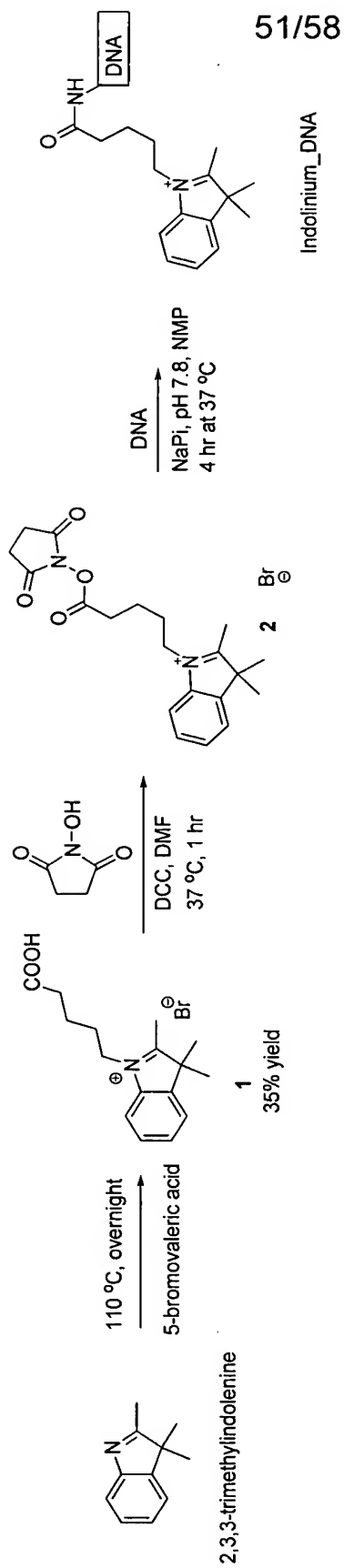
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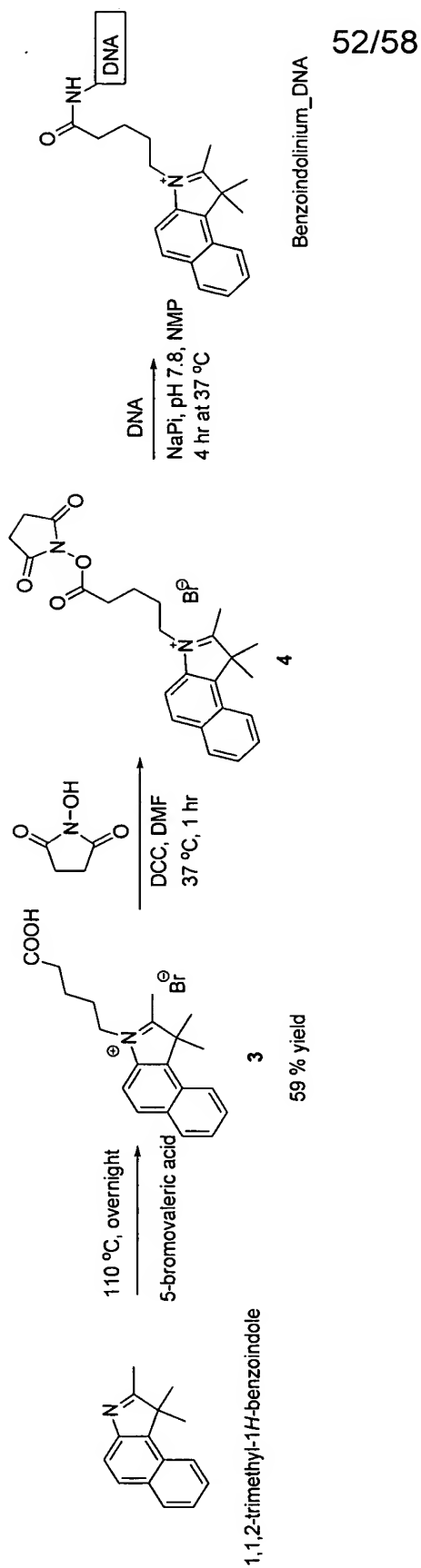
Scheme 1: Hemicyanine formation through organocatalytic aldol condensation in aqueous buffer.



Scheme 2: Hemicyanine dye generation through organocatalyst catalyzed DPC.

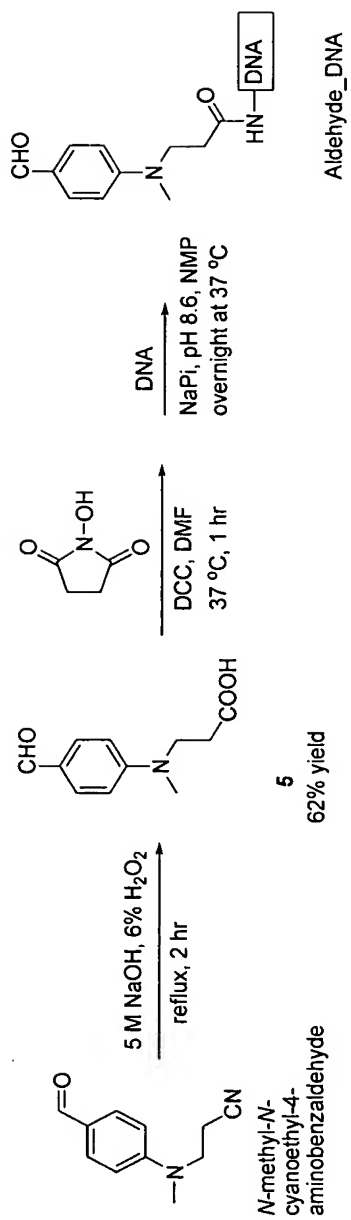


Scheme 3: Example of synthesizing DNA conjugated quaternary salt bearing active hydrogen component (indolinium_DNA).



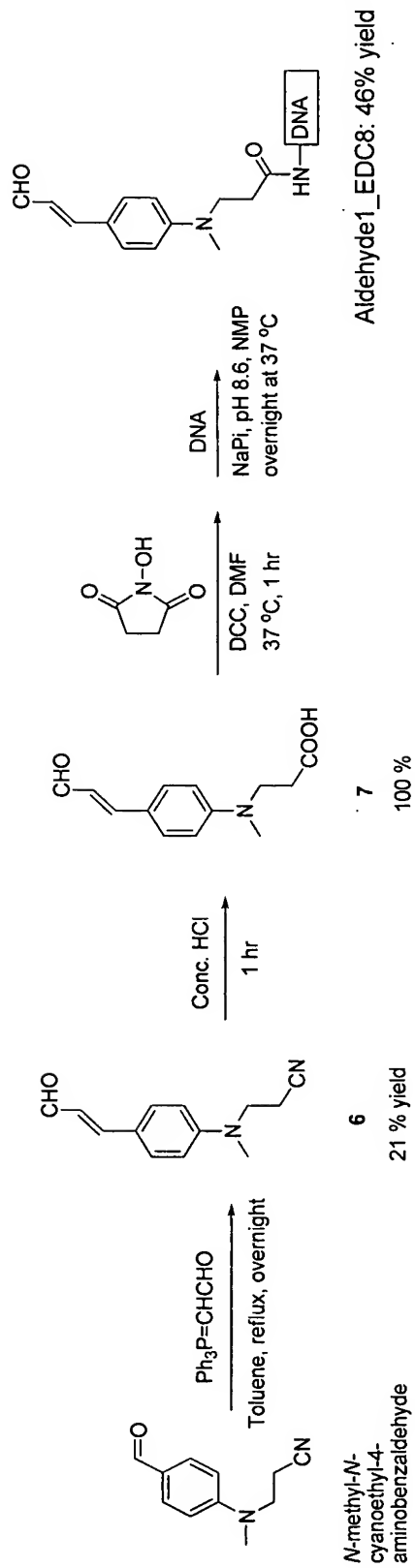
Scheme 4: Example of synthesizing DNA conjugated quaternary salt bearing active hydrogen component (benzoindolium_DNA).

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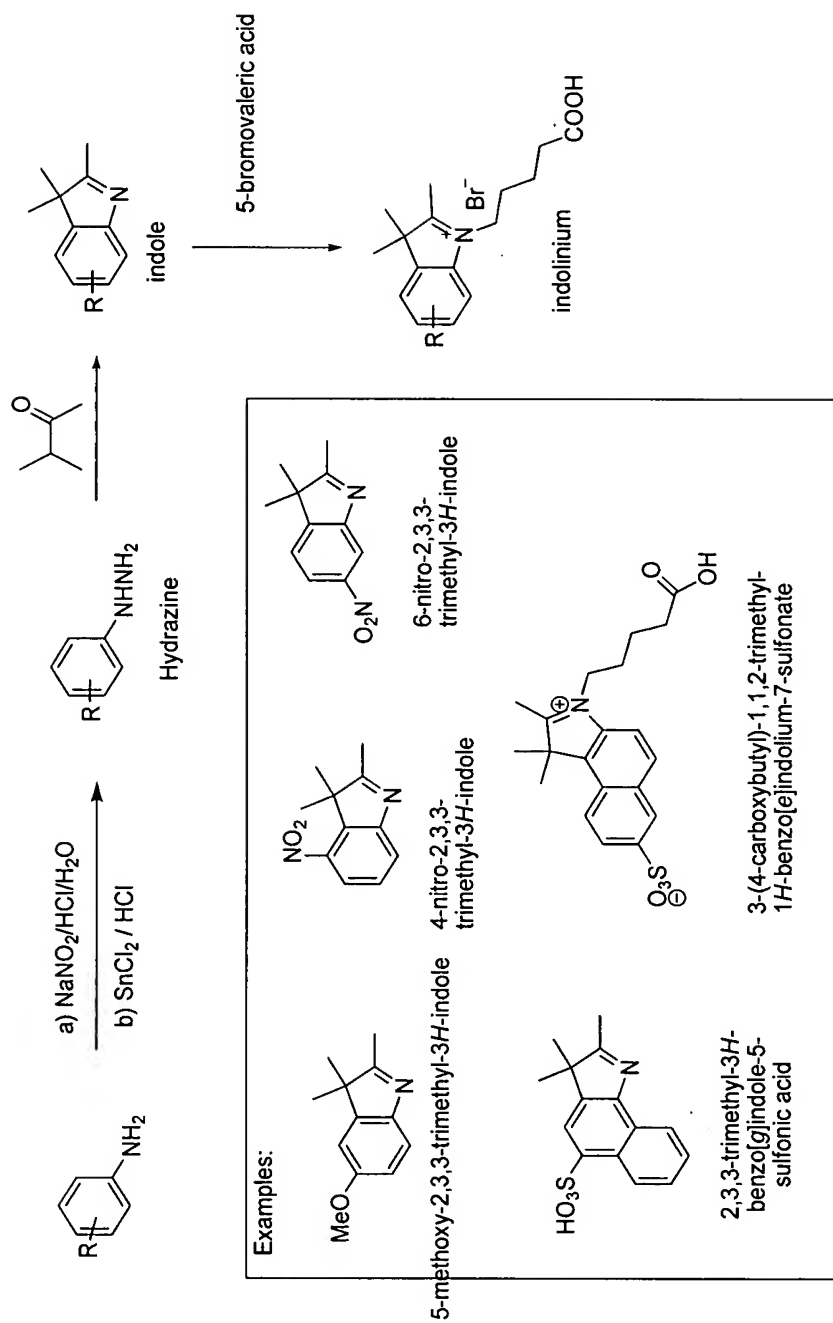
Scheme 5: Example of synthesizing DNA conjugated aldehyde (A0_DNA).

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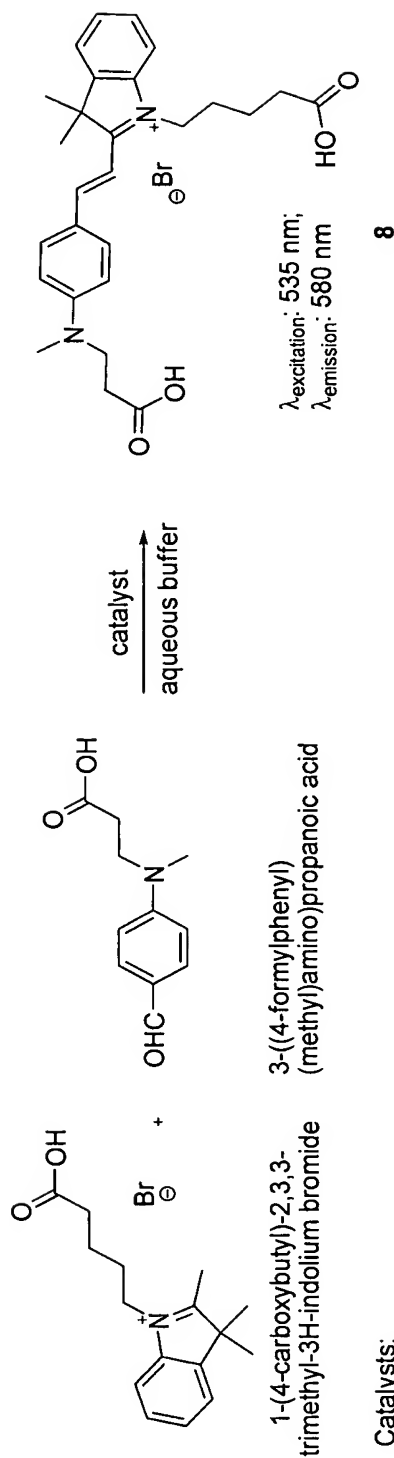
Scheme 6: Example of synthesizing DNA conjugated aldehyde (A1_DNA).

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Scheme 7: General synthetic routes to indole and indolinium analogues.

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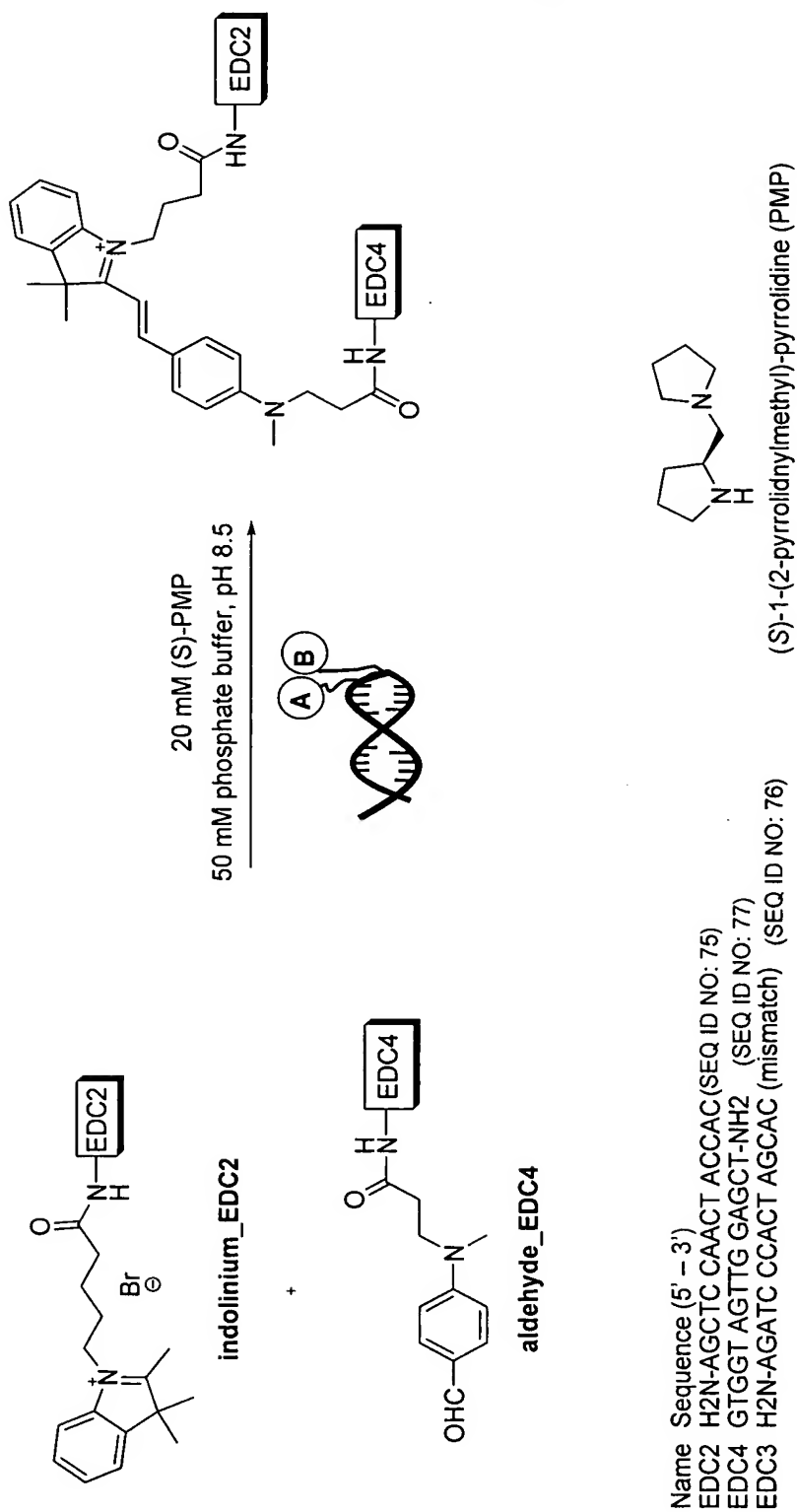
Catalysts:

- (1) L-valine (0.3 equiv. water); (2) L-proline (0.3 equiv. water);
 (3) (S)-Pyrrolidinemethylpyrrolidine (0.3 equiv. 50 mM sodium phosphate buffer, pH 8.5);
 (4) (S)-2-pyrrolidinemethanol (0.3 equiv. water); (5) Zinc-proline or Zn(Pro)₂ (0.1 equiv. 20 M sodium phosphate buffer, pH 8.5);
 (6) Pyrrolidine/acetic acid (0.4/0.2 equiv. water); (7) Pyrrolidine/10-camphorsulfonic acid (0.4/0.2, water)

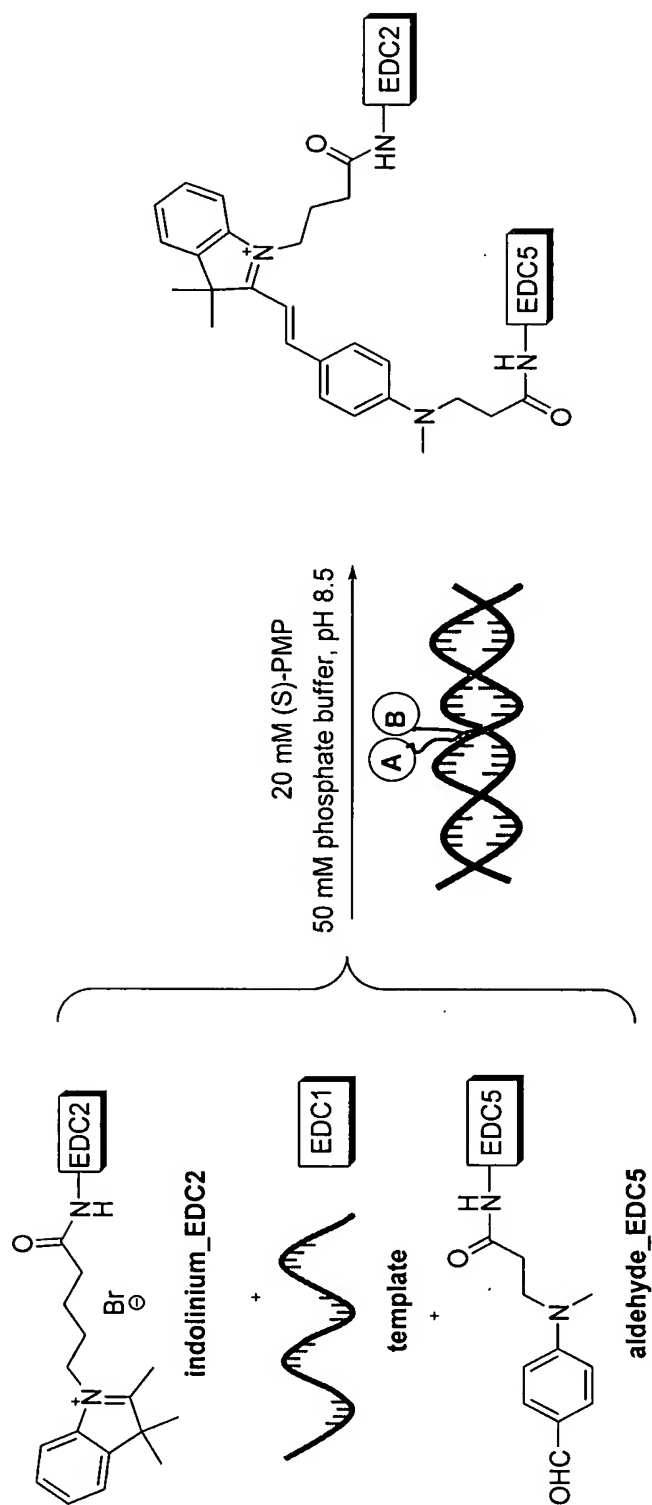
Reaction conditions: 25 mM each of the starting material, 20% NMP in water or sodium phosphate buffer plus required catalysts, RT for 16 hr.

Scheme 8: Hemicyanine formation in aqueous buffer in the presence of catalysts

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Scheme 9: Example of synthesizing hemicyanine_DNA dye 1 through DPC (end of helix).



Name Sequence (5' - 3')

name sequence (3'-5')
EDC1 GTGGT AGTTG GAGCT GTGG CGTAG GCAAGA (SEQ ID NO: 74)

EDC2 H2N-AGCTC CAACT ACCAC (SEQ ID NO: 75)

EDC5 TCTTG CCTAC GCCAC-NH2 (SEQ ID NO: 78)

Scheme 10: Example of synthesizing hemicyanine_DNA dye 2 through DPC (middle of helix).